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Applicants : Masakatsu KANEKO et al.

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DECLARATION UNDER 37 CFR 1.132

Commissioner for Patents
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Alexandria, VA 22313-1450

MAIL STOP AMENDMENT

S I R :

I, Makoto Koizumi, declare as follows:

A. My Education and Experience

1. I graduated from Hokkaido University, Sapporo, Japan, in the year 1986, and in 1991 I received a doctorate degree from Hokkaido University on the studies of catalytic ribozymes with sequence-specific RNA cleaving activity.

2. I have worked for Sankyo Company, Limited (now Daiichi Sankyo Company, Limited) of Tokyo, Japan, since 1991. My research activities at Sankyo Company, Limited have included the

following: synthesis of nucleoside analogs with antibiotic activity and synthesis of modified oligonucleotides with antivirus, anticancer and anti-diabetes activity. My research activities were not limited to those in the company; I studied as a visiting researcher at Yale University, New Haven, CT, for two years from November 1997. I presently hold the position of the Chief Researcher of Core Technology Research Laboratories of Sankyo Company, Limited. I have held this position since the year 2003.

3. I am a member of the Pharmaceutical Society of Japan. I am on the committee of Antisense DNA/RNA Society, Japan.

4. I have contributed many scientific papers. For example, I am a co-author of "Biologically active oligodeoxyribonucleotides. 5. 5'-End-substituted d(TGGGAG) possesses anti-human immunodeficiency virus type 1 activity by forming a G-quadruplex structure." *J. Med. Chem.* 1998, 41, 3655-3663; "Allosteric selection of ribozymes that respond to the second messengers cGMP and cAMP." *Nat. Struct. Biol.* 1999, 6, 1062-1071. Also, I am a corresponding author of "Synthesis and properties of 2'-O,4'-C-ethylene-bridged nucleic acids (ENA) as effective antisense oligonucleotides." *Bioorg. Med. Chem.* 2003, 11, 2211-2226; "Triplex formation with 2'-O,4'-C-ethylene-bridged nucleic acids (ENA) having C3'-endo conformation at physiological pH." *Nucleic Acids Res.* 2003, 31, 3267-3273; "Direct comparison of in vivo antisense activity of ENA oligonucleotides targeting PTP1B mRNA with that of 2'-O-(2-methoxy)ethyl-modified oligonucleotides." *Oligonucleotides* 2006, 16, 253-262.

5. I am named as an inventor in many patents issued in Japan, the United States and other countries. I am listed as an inventor in the following US patents: "Looped hairpin ribozyme." U.S. Patent 5,631,115 (issued May 20, 1997); "Modified oligodeoxyribonucleoditides." U.S. Patent 5,674,856 (issued

October 7, 1997); "Composition and method for the treatment or prophylaxis of viral infections using modified oligodeoxyribonucleotides." U.S. Patent 5,807,837 (issued September 15, 1998).

6. I was the recipient of the following academic and professional awards: "JB award, the Japanese Biochemical Society, 1996"; "Bioorganic Medicinal Chemistry Most Cited Paper 2003-2006 Award, 2006".

7. I am an expert in the way and manner of selecting and synthesizing compounds suitable for development as pharmaceuticals based on the chemistry and biology of nucleosides, nucleotides and oligonucleotides.

B. I am providing my opinion herein concerning the following:

1. What the publications discussed hereinbelow would teach a worker of ordinary skill in the art.

2. What a worker of ordinary skill in the art would understand the practical advantages to be of the superior activity and stability of ENA according to applicants' claims compared to BNA and Compound V of Wengel et al. USP 6,794,499 (hereinafter referred to as "Wengel et al.").

C. Characterization of a Person of Ordinary Skill in the Art

1. A person of ordinary skill in the art is a hypothetical person who is presumed to be aware of all the pertinent art.

2. A person of ordinary skill in the art is a person to whom an expert in the art could assign a task of moderate

difficulty, with reasonable assurance that the task would be accomplished without a great deal of supervision.

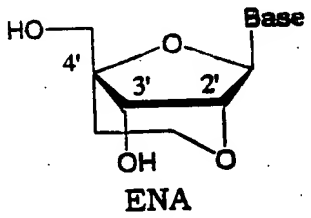
3. The following factors should be considered in determining who is a person of ordinary skill in the art: the types of problems encountered in the art; the prior art solutions to those problems; the rapidity with which innovations are made; the sophistication of the technology; and the educational level and experience of active workers in the field.

4. A person of ordinary skill in the art would have a graduate degree, such as a Master's Degree or a Doctorate Degree, in a field requiring knowledge of organic chemistry and/or biochemistry. The person with a Master's Degree should have at least five years research experience. The person with a Doctorate Degree should have at least two years of post-graduate academic or two years of post-graduate doctoral research in a company. The research experience of such a person should be in working for a pharmaceutical company in the development of pharmaceuticals. This person would have an understanding of the subject matter of the publications discussed herein.

D. The following statements of fact and opinion hereinafter, in my opinion, would be the statements of fact and opinion of a person of ordinary skill in the art identified hereinbefore.

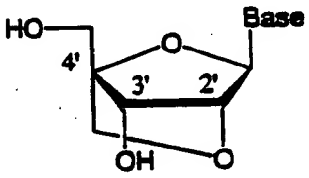
1. Applicants' claims are directed to ENA. As seen in the following structures, it is considered that the closest disclosed compounds in Wengel et al. are (LNA(BNA)). Compounds having an ethoxy bridge in the 2- and 3-positions (see Figs. 24 and 25 in Wengel et al.) are less closely related.

Applicants' Claims

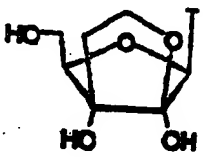


ENA

Wengel et al.



LNA (BNA)



2'-O,3'-C-CH_2CH_2 bridging group compound 13

2. The above compound 13, which is set forth in Fig. 25 of Wengel et al., has the same 2'-O,3'-C-ethylene bridge as compound V in Fig. 2 of Wengel et al. Compound V is described in Example 124 of Wengel et al. Some of the inventors of Wengel et al. published papers on the synthesis and properties of this compound V (Christensen et al., (1998), J. Am. Chem. Soc. 120, 5458-5463 and Nielsen et al., (1997), Chem. Comm. 825-826, copies of which are of record).

Compound V contains the same ethylene bridging group as the 2'-O,4'-C-ethylene bridged nucleic acids. However, in Christensen et al. supra, the authors said that the preferred furanose conformation of compound V has a pseudorotation angle *P* = 129° corresponding to the C1'-exo conformation. The pseudorotation angle *P* of the furanose of 2'-O,4'-C-methylene thymidine (an LNA unit) is 16.8° and 14.7° (the cell unit of 2'-O,4'-C-methylene thymidine has two conformers in a crystal structure analysis (Morita et al., (2003), Biorg. Med. Chem., 11, 2211-2226, a copy of which is of record). These *P* values correspond to a typical C3'-endo conformation. The pseudorotation angles *P* of the furanose of 2'-O,4'-C-methylene adenosine and 2'-O,4'-C-ethylene adenosine are 15.1 and 17.4, respectively, corresponding to the C3'-endo conformation (Morita et al. supra). These data indicate that two types of nucleosides containing a 2'-O,4'-C-methylene or a 2'-O,4'-C-ethylene bridging

group have a similar C3'-endo conformation and that compound V having a 2'-O,3'-C-ethylene bridging group has a different conformation than the nucleoside containing a 2'-O,4'-C-ethylene bridging group.

In Table 1 of Christensen et al. supra, melting temperatures of duplexes of oligonucleotides containing compound V units with complementary ssDNA or complementary ssRNA showed that these duplexes were less stable than the unmodified reference duplex. On page 825 of Nielsen et al. supra, it was stated that "incorporation of one to four modified bicyclic nucleosides X into a 14-mer destabilizes duplexes with the DNA complement dA14 by 2-3°C per modification." On the other hand, melting temperatures of duplexes of oligonucleotides containing a 2'-O,4'-C-ethylene bridging group with complementary ssDNA or complementary ssRNA showed that these duplexes were more stable than the unmodified reference duplexes (Morita et al. supra).

The melting temperatures of duplexes of oligonucleotides containing 2'-O,4'-C-methylene linkages have improved stability compared to the unmodified reference duplexes (see page 2212, left-hand column, lines 11 to 19 of Morita et al. supra). It should be further noted from Morita et al. supra that by contrast with the art, the nucleosides of the present claims having a 2'-O,4'-C-ethylene linkage give duplexes having even higher levels of stability (see page 2215, left-hand column, lines 11 to 14 of Morita et al., supra). It is respectfully submitted that this could not possibly have been predicted from Wengel et al.

3. The following publications are further evidence of the surprising improvement in properties achieved using the ENA compounds of the present claims when compared to the prior art compounds: Obika et al., (2001), Bioorg. Med. Chem., 9, 1001-1011; Koizumi et al., (2003), Nucleic Acids Research, 31, 3267-3273. Copies of Obika et al. and Koizumi et al. are of record.

In the aforesaid Obika et al. and Koizumi et al. publications, there are comparisons concerning the effect on triplex formation of incorporation into oligonucleosides of prior art nucleosides having a 2'-O,4'-C-methylene linkage and nucleosides of the present claims having a 2'-O,4'-C-ethylene linkage. Fully modified LNA oligonucleotides of the prior art did not bind to double-stranded DNA (see Obika et al. supra), whereas fully modified ENA oligonucleotides of the present claims have a high triplex forming ability (see Koizumi et al. supra).

The thermodynamic stability of the triplex containing ENA-3, a triplex-forming oligonucleotide ("TFO") fully modified with ENA (the melting temperature (T_m) value of ENA-3 was 42°C) was greater than that of a mixture of dsDNA and an oligonucleotide fully modified with LNA, BNA-3, which failed to bind to the dsDNA (see Table 1 in Koizumi et al. supra). A fully modified TFO, ENA-6, with 5-methylcytosine, instead of cytosine, also showed a much higher T_m value, 57°C, than that of a LNA oligonucleotide (BNA-6: T_m was not detected (see Table 1 in Koizumi et al. supra).

The binding activity of ENA oligonucleotides to dsDNA by gel analysis was investigated (see Figure 2 in Koizumi et al. supra). Each TFO was incubated with dsDNA in a ratio of 1:1 or 10:1 for 10 minutes at 60°C. After they were left for 60 minutes at room temperature, they were subjected to 10% PAGE with a neutral buffer at pH 7.2 at 20°C. In a ratio between TFO and dsDNA of 1:1, for fully modified ENA-6 only a faint band indicating triplex formation was observed (see Figure 2A in Koizumi et al. supra). In a ratio between TFO and dsDNA of 10:1, fully modified ENA-6 formed a triplex. However, the fully modified LNA oligonucleotide, BNA-6, completely failed to bind to dsDNA (see Figure 2B in Koizumi et al. supra).

A negative cotton effect was observed at approximately 215 nm in the CD spectra of the triplex. A cotton effect is the characteristic wavelength dependence of the optical rotary dispersion curve or the circular dichroism curve or both in the vicinity of an absorption band. In a ratio between dsDNA and TFO of 1:10, the negative cotton effect was observed at approximately 220 nm in the CD spectra of the complex with a fully modified ENA-6 (see Figure 3B in Koizumi et al. supra). In the case of a fully modified BNA-6, a negative band was not observed (see Figure 3B in Koizumi et al. supra).

The selected NF-κB binding sequences have a recognition site that is identified by restriction enzyme *Mln* I (see Figure 1B in Koizumi et al. supra). If a TFO binds to this recognition site of dsDNA, the *Mln* I reaction would be inhibited. At a pH of 7.2, each TFO was incubated with dsDNA in a ratio of 10:1 for 10 minutes at 60°C and left for 5 minutes at room temperature. This was followed by the addition of *Mln* I and incubation was carried out for 1 hour at 37°C. Finally, the resulting mixture was analyzed by denaturing 10% PAGE. Fully modified ENA-6 inhibited *Mln* I cleavage, but fully modified LNA TFO, BNA-6, did not (see Figure 4 in Koizumi et al. supra).

The above-described results demonstrate that fully modified ENA oligonucleotides can be used as TFOs, as opposed to the fully modified LNA oligonucleotides, which fail to form a triplex, as previously reported in Obika et al. It is respectfully submitted that this substantial difference in triplex-forming ability could not possibly have been predicted from Wengel et al. This improvement was demonstrated for oligonucleotides of the present claims having a variety of different bases at the 1'-position.

4. The Certificates of Experimental Results (2), (3), (5) and (6) attached to my September 2, 2006 DECLARATION UNDER 37 CFR 1.132 show that the ENA oligonucleotide had a remarkably higher

nuclease-resistance activity than an oligonucleotide containing BNA.

a. Stabilization against nucleases is essential for antisense agents and therefore many chemists have attempted to modify nucleosides. In this regard, see page 1630, right column, lines 14 to 18 of J. Kurreck, Eur. J. Biochem., 270, 1628-1644 (2003), a copy of which is of record, wherein the following is stated:

> "One of the major challenges for antisense approaches is the stabilization of ONs [oligonucleotides], as unmodified oligodeoxynucleotides are rapidly degraded in biological fluids by nucleases. A vast number of chemically modified nucleotides have been used in antisense experiments."

The word "stabilization" in the Certificate of Experimental Results (6) means a stabilization of plasma. "Stability in serum" in the Certificate of Experimental Results (3) and "stability in plasma" in the Certificate of Experimental Results (6) depend on nuclease-resistance. In this regard, see the enclosed copy of Arthur A. Levin, "A Review of Issues in the Pharmacokinetics and Toxicology of Phosphorothioate Antisense Oligonucleotides," Biochmica et Biophysica Acta, 1489, (1999), 69-84. The following which is stated in Levin in the paragraph bridging pages 73 to 74, refers to the stability of antisense oligonucleotides using phosphorothioate oligonucleotides with modified phosphate groups:

> "Unlike traditional low molecular weight drugs that are largely metabolized in cytochrome P-450-dependent pathways, phosphorothioate oligonucleotide drugs are metabolized by nucleases. The nature of the metabolites clearly delineates this pathway for metabolism. Capillary gel electrophoresis and LC-MS methods have demonstrated that there is a progressive shortening of the oligonucleotide. Specifically, mass spectral analysis of the metabolites shows the successive removal of bases from the 3' end of the

oligodeoxynucleotide is the major pathway for metabolic degradation in plasma."

That is, the main metabolic pathway of antisense oligonucleotides is the decomposition by a nuclease, and the decomposition of antisense oligonucleotides in plasma is promoted by a nuclease. Therefore, stability in plasma is a requirement for a useful antisense oligonucleotide.

b. Parallel triplex formation, which is discussed hereinabove, generally requires conditions of low pH (<6.0) necessary for protonation of the N3 of the third strand cytosine in the C+CG triplet.

Enclosed is a copy of Keith R. Fox, "Targeting DNA with Triplexes," Current Medicinal Chemistry, (2000), 7, 17-37, wherein the following is stated on page 32, left column, lines 2 to 7:

"One major problem for generating stable parallel triplexes under physiological conditions is the pH dependency of the C+GC triplet. although several analogues have been shown to alleviate this problem, none of these has found widespread use, possibly because of their limited availability, but also because of the large number of choices."

ENA is greatly superior to LNA (BNA) in triplex forming ability as described in paragraph D.3. hereinabove.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section

1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: May 7, 2007

Makoto KOIZUMI

Enclosures: (1) a copy of Arthur A. Levin, Biochmica et Biophysica Acta, 1489, (1999), 69-84

(2) a copy of A. Fox, Current Medicinal Chemistry, (2000), 7, 17-37

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Biochimica et Biophysica Acta 1489 (1999) 69–84

BIOCHIMICA ET BIOPHYSICA ACTA
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Review

A review of issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides

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Keywords: Antisense; Phosphorothioate oligonucleotide; Pharmacokinetics; Toxicology; Vitravene

Contents

1. Introduction

The development of antisense compounds as therapeutic agents is moving forward rapidly. The recent FDA approval of Vitravene (fomivirsen sodium) highlights the progress made in moving antisense oligonucleotides from the laboratory to the market place. Questions continually arise regarding the toxicity and pharmacokinetics of these novel therapeutic

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Table 1
Sequences of Isis compounds used in this article

Name	Sequence/linkages
ISIS 3521	GsTsTsCsTsCsGsCsTsGsGsTsGsAsGsTsTsTsCsA
ISIS 2105	TsTsGsCsTsTsCsCsAsTsCsTsTsCsCsTsCsGsTsC
ISIS 2922	GsCsGsTsTsTsGsCsTsCsTsCsTsTsCsTsTsCsTsTsGsCsG
ISIS 2302	GsCsCSCsAsAsGsCsTsGsGsCsAsTsCsCsGsTsCsA
ISIS 5132	TsCsCsCsGsCsCsTsGsTsGsAsCsAsTsGsCsAsTsT
GEM 91	CsTCsTsCsGsCsACsCsCsAsTsCsTsCsTsCsTsCsCsTsTsCsCsT

agents. This review will address some of the major areas of concern. Because most of the toxicology and pharmacokinetic data were generated with phosphorothioate oligonucleotides, this review will therefore concentrate on that class of antisense oligonucleotides. Sequences of all oligonucleotides referenced in this article are included in Table 1.

2. Pharmacokinetics issues

2.1. *Currently employed methods to deliver phosphorothioate oligonucleotides*

The initial clinical trials with oligonucleotides used local injections to achieve therapeutic concentrations at or near the disease site. For example, the first clinical trials of an antisense oligonucleotide were performed with intradermal injections of ISIS 2105 at the site of genital warts. ISIS 2105 is an agent targeting the translation start codon of the E2 mRNA of human papilloma viruses (HPV-6, HPV-11). Local concentrations of oligonucleotide at the site of injection were relatively high compared to concentrations known to have an antiviral activity in vitro, but there was also significant systemic absorption from intradermal injections [1,2]. In fact, widespread systemic absorption has been documented following both intradermal and subcutaneous injections [1,3–5].

Local delivery at the site of viral infection was successfully employed with fomivirsen, a 21-mer oligonucleotide that is complementary to a sequence in an immediate early gene of human cytomegalovirus (hCMV). This compound inhibits hCMV replication and intravitreal injections have been proven to be effective in the treatment of CMV retinitis in patients with AIDS. In contrast to the widespread distribution of oligonucleotide following intradermal administration, after intravitreal injections, little or no systemic exposure to the oligonucleotide could be demonstrated in animals treated with clinically relevant doses, nor was there detectable oligonucleotide in the plasma of treated patients (unpublished observation, Isis Pharmaceuticals). This limited systemic availability was an asset for this drug because only small amounts of drug need to be injected in order to achieve therapeutic concentrations. Additionally, the local dose was not diluted in the larger depots for oligonucleotides that are present in other organs. Thus, in the case of fomivirsen, the absence of systemic absorption was an important factor in the drug's development.

In more recent clinical trials, antisense oligonucleotides have been administered by subcutaneous injections, or subcutaneous or intravenous infusions. Following intravenous or subcutaneous injections, phosphorothioate oligonucleotides are cleared from plasma and are distributed to a variety of organs [1,6]. Distribution from plasma to organs is more rapid than is metabolism and significant concentrations of intact drug reach numerous target organs (see below).

2.2. *Delivery of phosphorothioate oligonucleotides: recent progress*

There are practical limitations to drug administration by intravenous infusions and because of these, there are extensive efforts to make delivery of antisense oligonucleotides more convenient. Recent studies have demonstrated that there are promising technologies for administering antisense compounds without intravenous infusions. Using proprietary

topical formulations, it is possible to expose epithelium and the dermis to phosphorothioate oligonucleotides. Immunohistochemistry studies have also been able to localize oligonucleotide in various important cell types in the epidermis and dermis. Obviously, topical application of antisense oligonucleotides would be a favored route for employing antisense molecules in the treatment of dermatologic disorders (unpublished observation, Isis Pharmaceuticals). We are currently characterizing the pharmacokinetics and safety profiles of dermally applied phosphorothioate oligonucleotides.

Topical administration of oligonucleotides to the gut epithelium is also being explored. Recently completed toxicity studies with ISIS 2302 in an enema formulation indicated that it was possible to expose the gut epithelium to oligonucleotide. Four weeks of treatment with up to 50 mg/kg rectally was well tolerated locally with no systemic toxicities observed. There was some small amount of systemic exposure but total systemic bioavailability was less than 5% (unpublished observation, Isis Pharmaceuticals). Using other formulations it has been possible to markedly improve the bioavailability of oligonucleotides from the gut. These results indicate that with the appropriate oligonucleotide chemistries and formulations it should be possible to have orally available antisense compounds (unpublished observation, Isis Pharmaceuticals). Preliminary data with formulated oligonucleotides indicate that following intrajejunal installation absolute bioavailabilities of upward of 15% can be achieved, indicating that it should be possible with the appropriate release of drug and permeability enhancer in the gut to have orally active antisense drugs. Studies on the pharmacologic activity and the pharmacokinetics of orally administered oligonucleotides are in progress. Earlier studies performed without excipients have yielded very low levels of bioavailability [7], again suggesting the need for more sophisticated dosage forms.

Inhalation is also being explored as a possible route of administration. Using aerosols of oligonucleotide solutions it has been possible to attain significant pulmonary concentrations of oligonucleotides in the lung. Following inhalation exposure, oligonucleotide is found widely distributed within the lung in both the airways and in the terminal alveoli. Using immunohistochemistry, it is possible to show oligonucleotide in a number of cell types included pulmonary macrophages, type 1 and type 2 pneumocytes and endothelium. Preliminary results indicate that uptake by the lung is saturable and that phosphorothioate oligonucleotides are cleared from the lung by a combination of metabolism and distribution to other tissues. The half-life of phosphorothioate oligonucleotides in lung is on the order of 20 h and thus it appears that the pharmacokinetics of pulmonary delivery are amenable to local therapy. Inhaled doses which produce local concentrations of oligonucleotide known to produce pharmacologic effects have little or no local responses after limited courses of treatment, suggesting that there could be a significant therapeutic index (Templin et al., manuscript submitted).

Intratracheal administration of oligonucleotides is often used as a model for inhalation exposure. Intratracheal administration of a phosphorothioate oligonucleotide demonstrated: (1) that the drug was well tolerated by the lung and (2) that it was possible to achieve systemic exposure following local treatment of the lung. However, the preliminary data obtained in our laboratories have demonstrated a relatively low level of systemic exposure following intratracheal administration of phosphorothioate oligonucleotides (unpublished observation, Isis Pharmaceuticals). Thus, it remains to be determined whether inhalation of oligonucleotides is a viable route of administration for diseases of other organs outside the lung.

2.3. *Plasma kinetics following systemic administration*

Systemic delivery of oligonucleotides has been achieved by subcutaneous injections, or intravenous or subcutaneous infusions. In clinical trials employing intravenous infusions of 2 h duration, the plasma is cleared of oligonucleotide with a half-life in the range of 0.5–1 h depending on dose [8]. Phosphorothioate oligonucleotides circulate in plasma bound to plasma proteins that protect them from filtration. When chemical modifications to the backbone are made such that protein binding is reduced, then there is a marked increase in urinary excretion of the modified oligonucleotide [9].

Rapidly after the initiation of these infusions it is possible to detect oligonucleotide metabolites in plasma. However, metabolism is not the primary mech-

anism of plasma clearance, based on the observation that the parent compound is almost always present even after two to three half-lives. Studies in animals indicate that distribution of the oligonucleotide into tissues is the predominant driving force in plasma clearance [1,5,6,10–15].

Plasma kinetics are non-linear and the area under the concentration versus time curve (AUC) increases with dose greater than would be predicted on the basis of dose alone. As the dose of ISIS 2302 was increased from 0.5 mg/kg to 2.0 mg/kg, AUC increased approx. 6-fold [8]. A similar non-linear relationship between dose and AUC was observed in animal studies with ISIS 2302 and other phosphorothioate oligonucleotides. Because AUC is related to plasma clearance, and plasma clearance is primarily a function of distribution to tissues, we suggest that the non-linearities in plasma kinetics may be secondary to dose-dependent changes in organ kinetics (see below). However, the most important pharmacokinetic parameters are tissue half-life or mean residence time. Antisense oligonucleotides produce activity by interacting with mRNAs in target tissues. Therefore, the most important kinetic parameters relate to this interaction. In the case of clinical trials, the inability to measure target organ concentrations forces us to rely on extrapolations from preclinical studies and what is known about the relationships among dose, plasma AUC and tissue concentrations.

Clinical trials are also being performed using subcutaneous injections. Preliminary data from these studies, and from animal studies, indicate that there is significant systemic exposure following s.c. injections. Subcutaneous injections produce attenuated peak concentrations of the oligonucleotide compared to i.v. injections and the plasma AUC for s.c. injections is roughly half that of an equivalent dose given by a 2 h intravenous infusion. Despite the differences in the plasma kinetics between equivalent i.v. or s.c. doses, the concentrations in target organs were almost equivalent, suggesting that total systemic exposure was similar (Leeds et al., manuscript submitted). This finding may be explained by either differences in organ uptake with differing plasma profiles (less saturation at lower plasma concentrations) or by uptake from the site of injection other than the plasma compartment, (e.g. lymphatic uptake) or a combination. In light of high local concentrations in the lymph nodes draining the sites of injection, it appears that lymphatic uptake is at least a component in the total systemic exposure following subcutaneous injections [5,16].

2.4. Distribution to organs following systemic absorption

Oligonucleotides are detected in nearly all tissues and organs within minutes of intravenous, subcutaneous and intradermal doses. The exceptions to the broad tissue distribution include brain and testes that appear to have significant barriers for the transport of these types of molecules and they are not target sites for direct toxicity.

Following subcutaneous, intravenous, and intradermal administration, the liver and kidney are the major sites of accumulation of phosphorothioate oligodeoxynucleotides with the spleen, bone marrow and lymph nodes having somewhat lower levels of oligodeoxynucleotide. Other tissues and organs accumulate some levels of oligonucleotide but not to the same extent as liver, kidney, and spleen. In a segment 2 teratology assay, intravenous injections of oligonucleotides to pregnant mice during gestation results in exposure of the placenta, but little or no accumulation was observed in fetal tissues (unpublished observation, Isis Pharmaceuticals).

With phosphorothioate oligonucleotides, the organ distribution appears to be relatively independent of sequence. As antisense oligonucleotide chemistry diverges from the unmodified phosphorothioate backbone and as the ribose is modified, the organ distribution (and the subcellular distribution) may change. Crooke et al. have demonstrated that by altering chemistry it is possible to alter protein binding and organ distribution. These properties can be utilized to design compounds that target specific organs and cell types [9].

2.5. The relationships between tissue distribution and plasma kinetics

Plasma kinetics are not linear with dose and this non-linearity may be related to organ distribution. Plasma concentrations increase proportionally to dose, but the increases are greater than would be predicted based on dose alone [14,17–20]. Part or

all of the non-linearity in plasma kinetics may be related to the saturation of tissue uptake. Liver and kidney concentrations of oligodeoxynucleotide appear to saturate as dose is increased. Renal concentrations of oligonucleotides also appear to saturate increasing only 4-fold over a 10-fold dose range, but the degree of saturation is not as apparent as that in the liver. Preliminary data suggest that the degree of saturation observed at these dose levels would be altered if the doses were infused more slowly over longer periods of time. In fact, prolonging the infusion times allows for increased accumulation by liver and kidney, suggesting that the non-linearity is both a function of dose rate as well as dose (Geary and Levin, unpublished observation). These saturation phenomena have significant implications for understanding the relationships between dose and toxicity. If tissue saturation modulates plasma pharmacokinetics, then it is possible that changes in the organ distribution could ultimately affect plasma concentrations and alter the predictable patterns now established between dose and peak plasma levels.

The metabolism and rate of clearance of oligodeoxynucleotides determines tissue accumulation, and ultimately may influence toxicity. In rats and monkeys, phosphorothioate oligodeoxynucleotides are cleared from tissues and organs by nuclease-mediated metabolism with half-lives that vary from 20 to 120 h depending on the tissue or organ [1,6,21]. Significant accumulation of oligodeoxynucleotide in liver and kidney after repeated doses results from the extensive partitioning into tissues, combined with the relatively slow metabolic clearance rates. In some tissues, there is accumulation with repeated administration even after every-other-day dosing. Thus, many toxicity studies with phosphorothioate oligodeoxynucleotides have employed every-other-day dosing regimens. Multiple-dose kinetics are similar to single-dose kinetics and metabolite patterns are similar to those in single-dose studies. Repeated dosing neither induces, nor inhibits, metabolism. There are no alterations in plasma half-life or kinetics at low doses. At higher doses, saturable kinetics are apparent and there are some changes in tissue distribution [22].

The long tissue half-lives for liver and kidney result in significant exposure of these organs. Because of this accumulation, these are likely target organs for toxicity. In fact, histopathologic and functional changes have been observed in these organs in high-dose animals at the end of subchronic and chronic dosing (see below).

2.6. Distribution into cells

Oligonucleotides must be internalized into target cells in sufficient amounts to inhibit expression of the targeted RNA. At early times, up to 2 h after parenteral administration, oligonucleotides are associated with extracellular matrix as well as localized intracellularly [23]. At later times, increasing amounts of the oligonucleotide are found inside cells. Antisense oligonucleotides do not distribute uniformly within tissues but accumulate within certain cell populations, but the data are unequivocal that phosphorothioate oligonucleotides enter cells and can be found in various subcellular compartments. In fact, there may be distinct patterns of subcellular distribution with different oligonucleotide sequences (R. Crooke et al., unpublished observations).

It appears that binding to proteins is an important factor in cellular uptake. There are a number of hypotheses to explain the movement of oligonucleotides into cells. Cells take up oligonucleotide in an energy-, temperature-, and time-dependent process. Pinocytosis or podocytosis has been recognized as a potential mechanism of cell uptake. Macrophages, Kupffer cells, proximal tubular cells, and other cells that are active phagocytically have all been shown to concentrate oligonucleotides [13,23,24]. But cellular uptake is not solely the domain of phagocytes; other cell types take up oligonucleotides too. Hepatocytes, vascular endothelial cells, bone marrow cells, splenocytes, and other cell types in the skin all take up oligonucleotide after in vivo dosing. The mechanism of the cell uptake, like other drug classes, has not been fully elucidated, but there may be additional mechanisms of transmembrane movement in addition to pinocytosis.

2.7. Mechanism of metabolism

Unlike traditional low molecular weight drugs that are largely metabolized in cytochrome *P*-450-dependent pathways, phosphorothioate oligonucleotide drugs are metabolized by nucleases. The nature of

the metabolites clearly delineates this pathway for metabolism. Capillary gel electrophoresis and LC-MS methods have demonstrated that there is a progressive shortening of the oligonucleotide. Specifically, mass spectral analysis of the metabolites shows the successive removal of bases from the 3′ end of the oligodeoxynucleotide is the major pathway for metabolic degradation [25–27] in plasma. Both 5′ and 3′ exonuclease excision may occur in tissues. 3′ end shortened metabolites are more common than 5′ shortened metabolites. Endonuclease mediated degradation of phosphorothioate oligonucleotides is not generally observed. Oligonucleotides can be protected from exonuclease mediated activity by placing functional groups at the 2′ position of the ribose sugar. When one modifies an oligonucleotide at the 3′ or 5′ ends, but leaves the central portion with the diester linkages unmodified, it is possible to observe metabolites characteristic of endonuclease metabolism. For these compounds, the pattern of metabolism differs from the laddering observed with progressive removal of single nucleotides. The pattern of metabolism with these compounds is such that there are a few prominent peaks representing the oligonucleotide fragments being cleaved at susceptible sites by endonucleases.

Exonuclease metabolism in plasma and tissues is rapid. Almost immediately after intravenous administration of phosphorothioate oligonucleotides it is possible to measure chain-shortened metabolites of the parent compound. As early as 5 min after administration 30–40% of the total oligonucleotide has at least a single nucleotide deleted. This percentage only increases slightly with time and the parent compound is generally the most abundant oligonucleotide for up to 4 or more hours [8,22]. Hours after dosing rodents with oligodeoxynucleotide, metabolites shortened from either the 3′ or 5′ ends can be identified using electrospray mass spectral analysis [28]. Similar oligonucleotide metabolites were detected in tissues (liver and kidney) following subcutaneous administration of phosphorothioate oligodeoxynucleotides [29,30].

The resultant products of nuclease degradation are nucleotides either with a phosphorothioate or phosphodiester group. From metabolism studies with [^{14}C]thymine labeled at the C-2 position, it appears that the ultimate metabolic fate of the liberated nucleotides is via a metabolic pathway similar to that for endogenous nucleotides. Thus, CO_2 is the final product of the C-2 labeled thymidine and presumably other nucleotides from phosphorothioate oligodeoxynucleotides are similarly metabolized. These same patterns of single base excision and chain-shortened metabolites have been reported for all species from mouse to man, suggesting that the metabolic pathways are similar. When phosphorothioate oligonucleotides are radiolabeled with ^{35}S in the thioate linkage, the pattern of excretion of radiolabel changes. The radiolabel in this case is labile and ends up being metabolized down to low molecular weight sulfur containing products that are excreted in the urine [10]. The nature of these metabolites is currently under investigation, but oxidation of the phosphorothioate linkage likely results in the liberation of inorganic sulfate or thiophosphate.

3. Toxicity issues

Toxicity from antisense oligonucleotides can be induced by hybridization-dependent and hybridization-independent mechanisms. Major issues in characterizing the toxicity of antisense compounds are to differentiate between the hybridization-dependent and -independent toxicity and how to insure that there are no inappropriate hybridization events. Careful analysis of the existing data provides insight into these issues. The toxicities observed in preclinical toxicity studies all appear to be related to the chemical class, phosphorothioate, and are generally independent of sequence-related effects. The only sequence-related toxicity observed to date is the immunostimulatory effects discussed below.

3.1. Sequence-dependent effects that are unrelated to RNA hybridization

Oligonucleotides can produce pharmacologic effects that are independent of antisense activity [31–35]. For example, guanine quartets can produce distinct pharmacologic effects [36] resulting from either protein binding or binding non-specifically to RNA. This strategy of oligonucleotides containing G-quartets to bind to proteins has been used to block the infectivity of HIV. Studies in vitro demonstrated that

oligonucleotides with this type of structure could block HIV binding to target proteins on cells [37].

Hybridization-independent effects of oligonucleotides have also been attributed to specific sequences that can induce immunologic responses. This immunologic response has been generally described as immune stimulation and has been associated with a specific sequence motif: a CpG motif in which CG residues are flanked by two purines at the 5′ and two pyrimidines 3′ (e.g. AACGTT). This motif has been shown to be particularly active at inducing B-cell proliferation and the release of cytokines [35,38,39]. Oligonucleotides, particularly phosphorothioate oligonucleotides, can also activate NK cells [40,41]. Treatment of mice in vivo with these oligonucleotides containing these motifs can produce very profound immune stimulation including marked splenomegaly [39,42]. Rodents appear to be more sensitive to this effect compared to primates.

The response of rodents to treatment with phosphorothioate oligodeoxynucleotides is characterized by a dose-dependent splenomegaly, lymphoid hyperplasia, and diffuse multi-organ mixed mononuclear cell infiltrates. At doses above 10 mg/kg the liver, kidney, heart, lung, thymus, pancreas and other tissues have infiltrates of monocytes, lymphocytes, and fibroblasts [4,42–44]. Normal cellular architecture is maintained, and fibrotic changes are NOT observed as would be expected in a classical inflammatory response. The infiltrate was partially or fully reversible upon the cessation of treatment [4,43].

The spleens of treated mice show evidence of B-cell hyperplasia, histiocytosis in the connective tissue capsule and stromal hyperplasia. There are increases in B-cells as well as blast forming cells and granulocyte-monocyte forming cells [39]. Other lymphoid organs were also enlarged by treatment. In vivo and in vitro data suggest that the mitogenic effects are primarily on B-cells. These immunostimulatory effects are associated with the release of cytokines both in vitro and in vivo, IL-6, IL-12 and interferon γ [35,38,45,46]. Oligodeoxynucleotide treatment can also stimulate cytokine production by other cell types. For example, primary cultures of human keratinocytes secrete IL-1α in response to treatment with a phosphorothioate oligodeoxynucleotide [47]. It is the secretion of these cytokines that may ultimately induce the infiltration of mononuclear cells that has been such a prominent feature in toxicity studies in rodents.

The nature of the immunostimulatory motifs follows a complex set of rules. Monteith et al. showed that splenomegaly occurs to some extent whether or not there is a CG in the sequence, suggesting that these motifs are not the sole determinants of immune stimulation [42]. Clearly, CG and palindromic elements containing CG are important in the potent stimulation of both B-cell proliferation, as well as cytokine release. High affinity receptors have been proposed that might control these interactions [48], but others have proposed that the oligonucleotide must be internalized for maximal proliferative effect [49–51]. Obviously, the identification and structural mapping of high affinity binding sites on or in the lymphocytes will aid in the understanding of this interaction.

Rodents are highly susceptible to this generalized immune stimulation, whereas primates appear to be relatively insensitive to the effect at equivalent doses. The mixed monocellular infiltrates in liver and other organs that are so characteristic of the response in rodents are absent even after long term exposure in monkeys [3,52]. The pharmacokinetics of phosphorothioate oligodeoxynucleotides in rodents and primates are similar suggesting that the species differences in immunostimulatory effects are not the result of differences in plasma kinetics or tissue distribution. From the existing data we conclude that rodents are markedly more sensitive to phosphorothioate oligodeoxynucleotide-induced immune stimulation than primates. There is an active series of investigations attempting to characterize whether oligonucleotides can be used as adjuvants for vaccines and cancer therapy [51,53–56].

3.2. *Immune responses directed toward oligonucleotides*

While it is well known that oligonucleotides induce B-cell proliferation and stimulate antibody production, there is no evidence that oligonucleotides are themselves antigenic. A guinea pig maximization test yielded negative results with ISIS 2105, an unmodified phosphorothioate oligonucleotide complementary to an mRNA in human papilloma virus. The same oligonucleotide was administered to mon-

keys and rats treated by intradermal injections every other day for 4 weeks. Serum was analyzed for the presence of antibodies to ISIS 2105. As would be predicted from the B-cell stimulatory effects, there was a very slight increase in total antibodies, but no indication of a significant increase in anti-ISIS 2105 antibodies (Leeds and Truong, unpublished data).

There were no detectable IgG or IgM antibodies specific to ISIS 2302 in human volunteers treated with ISIS 2302 (Leeds, Truong and Levin, unpublished data). Thus, we conclude that treatment of rats, monkeys, or humans with phosphorothioate oligodeoxynucleotides does not result in anti-oligodeoxynucleotide antibodies, but we are continuing to monitor for this response preclinically and clinically. There has been no indication of delayed type hypersensitivity in the animal studies.

3.3. *Factors that make toxicity from unintended antisense activity toward non-target RNA improbable*

The goal of antisense therapy is a temporary 'knockdown' of the target gene expression by inhibiting the translation of key gene in a disease process. It is also possible that reductions in gene expression will result in some undesirable biologic effect. In this case the adverse effect would be associated with the intended antisense mechanism. To test for this type of toxicity in the preclinical setting we have administered oligodeoxynucleotides that were directed to the target mRNA in that animal species. By careful selection of target mRNAs we have not observed sequence-dependent toxicity. For obvious reasons, gene targets that would dramatically affect cell viability have been avoided.

Theoretically, toxicity might also be induced through hybridization of the antisense agent and a mRNA that was not the intended target. This type of event should be exceedingly rare because of the specificity encoded into the antisense molecule. Specificity is a function of the number of bases such that with an oligodeoxynucleotide 15–21 bases in length, there is little probability that hybridization with an unintended target could occur. In practice this probability is diminished even further, given the fact that hybridization affinity decreases significantly if there are mismatches, and is further reduced because only a fraction of perfectly matched antisense oligodeoxynucleotides have significant pharmacologic activity.

Comparing the activity of different 20-mer oligodeoxynucleotide sequences that hybridize with different sites along entire genes from 3′ untranslated regions to 5′ untranslated regions demonstrates that there are marked differences in the antisense activity of different sequences. In fact, many if not most of the oligodeoxynucleotides in this type of 'gene walk' are relatively weak inhibitors of gene expression [57–63]. This apparent fastidiousness for specific target sites may be related to accessibility of the targeted mRNA sequence to the antisense construct [57,59,64–69]. Only sites on mRNA that are accessible to the antisense molecule will be potential sites for antisense activity. Depending on the specific mRNA and its secondary structure, there may be a limited number of ideal (or active) target sites for antisense inhibition. This accessibility factor further reduces the probability that there would be unintended hybridization. In addition, other studies have demonstrated that there is a significant loss of pharmacologic activity when the antisense compound has one or more mismatched bases suggesting that potent pharmacologic activity requires near perfect sequence matches. There has been concern that smaller metabolites of antisense oligonucleotides begin to lose specificity as they are reduced in length. It should be noted that at the same time that oligonucleotides are losing specificity they are also losing binding affinity that will limit binding. Finally, there is concern that because some sequences in the human genome are complementary to metabolites of antisense oligonucleotides there could be unintended hybridization. If the unintended hybridization to some homologous sequence is going to produce toxicity then ALL of the following must be true: (1) the sequence must be expressed, (2) there must be a perfect or near perfect sequence match to the antisense molecule or its metabolite, (3) the site of perfect homology must be accessible to the antisense oligonucleotide and (4) reduction in the expression of the unintended target is incompatible with normal function. Taken together these factors suggest that the probability of toxicity occurring as a result of unintended cross-hybridization is vanishingly small.

3.4. Class-related toxicities of phosphorothioate oligonucleotides

While toxicity is unlikely to be the result of hybridization to unintended targets, there are other potential mechanisms of toxicity unrelated to the antisense mechanism. Antisense therapeutic agents, like all other xenobiotics, have their own inherent toxicities that stem from the physical or chemical characteristics of the compounds. In contrast to toxicities mediated by antisense mechanisms, these toxicities would be generally less dependent on sequence and more dependent on the chemical class. The class-related toxicities are mediated through mechanisms other than hybridization, for example oligodeoxynucleotide-protein interactions. Most, if not all, toxicities observed in preclinical studies are class-related toxicities.

The phosphorothioate oligodeoxynucleotides are water soluble, polyanionic molecules with a length of 20–25 nucleotides. These compounds are known to bind to proteins with affinities that span the micromolar to the millimolar ranges [70,71]. These protein-binding affinities suggest that at plasma levels attained in toxicity studies there can be significant interactions with proteins. Note that if the physical and chemical properties of phosphorothioate oligodeoxynucleotides are the predominant driving forces in these protein interactions, then sequence or length would not be particularly strong influences on toxicity because small changes in sequence and length have little effect on the physical chemical properties of an antisense agent. In fact the results from preclinical toxicity studies support this hypothesis. All toxicities observed in preclinical studies are independent of hybridization and are dependent on protein interactions.

These non-antisense mediated pathways are responsible for most, if not all, of the toxicities associated with the administration of these compounds to laboratory animals. In support of this conclusion, little or no difference in toxicity is observed between pharmacologic active and inactive sequences [3,4,43,44,72].

Although there are similarities in the patterns of toxicologic response that are independent of sequence there are occasional differences in the relative potency between sequences. This spectrum of potency is thought to be due to specific sequence motifs that might enhance the protein binding and ultimately toxicity. To date, however, only quantitative differences exist and qualitatively the toxicities are similar [4,43,44,72,73]. Thus, there is an apparent paradox regarding toxicities that are generally independent of sequence hybridization but that may be enhanced by specific sequences like the immunostimulatory effects of oligonucleotide discussed above.

Similarities in toxicity between sequences are exemplified by acute toxicity data. The doses of three phosphorothioate oligodeoxynucleotides required to produce 50% lethality were estimated to be approx. 750 mg/kg. With relatively high LD_{50} values, these compounds appeared to have the same low potential to induce acute toxicity in mice. Studies in primates have demonstrated that the potential for acute toxicity appeared to be markedly different from rodents but still similar from compound to compound.

In primates, the most serious acute toxicity is the result of a transient activation of the complement cascade [74–76]. After rapid infusions and at relatively high plasma concentrations activation of the complement cascade has in some instances led to cardiovascular collapse and death. This toxicity is related to peak plasma concentrations and can be controlled by limiting plasma concentrations. This toxicity, as well as an inhibition of the clotting cascade, is thought be related to the polyanionic nature of the molecules and the binding of these compounds to specific protein factors in plasma.

Complement activation is relatively common when plasma concentrations exceed 40–50 μg/ml of oligonucleotide. However, in a small fraction of the total instances when complement activation occurred, we observed marked hematologic effects and marked hemodynamic changes. In these instances, complement activation was accompanied by marked reductions in heart rate, blood pressure and subsequently cardiac output that are lethal in some animals [74,76,77]. Hematologic changes are characterized by transient reductions in neutrophil counts presumably due to margination, followed by neutrophilia with abundant immature non-segmented neutrophils (bands). The increase in immature cells is probably the result of neutrophils being recruited by the release of chemotactic factors.

All treated monkeys demonstrating some degree of

cardiovascular collapse or hemodynamic changes had markedly elevated levels of complement split products. However, the converse is not true, in that only a fraction of the animals with activated complement had cardiovascular functional changes. There may be sensitive subpopulations or predisposing factors (like stress) within individual animals that make them susceptible to the physiologic sequelae of complement activation.

Complement activation was characterized by reduction of serum hemolytic potential (CH_{50} analysis) and concomitant increases in the liberation of complement split products Bb, C3a, and C5a, but no increase in C4a. The absence of C4a and the presence of Bb are consistent with the activation of complement occurring through the alternative pathway.

The exact mechanism for complement activation is unknown but there are some potential explanations that need to be further explored. The alternative pathway is constitutively active, but under most circumstances the low level of activity is retained in control by the circulating negative regulatory proteins, Factor H and Factor I. Interactions of phosphorothioate oligodeoxynucleotides with Factor H may play a role in the activation [76]. Reductions in Factor H produced by another polyanion, dextran sulfate, have been reported to activate the alternative pathway in vitro [78]. Deactivation of a regulatory factor could lead to uncontrolled activation of the alternative complement cascade similar to that described in monkeys treated with high doses of phosphorothioate oligodeoxynucleotides. Phosphorothioate oligodeoxynucleotides bind to Factor H [76] and in some way this may result in its deactivation or sequestration.

The complement cascade is activated only at high plasma concentrations of phosphorothioate oligodeoxynucleotides and by controlling the dose and rate of infusion, it is possible to administer phosphorothioate oligodeoxynucleotides in such a way as to avoid complement activation. The toxicity associated with complement activation is probably secondary to the physiologic responses to the formation and release of biologically active complement split products C3a and C5a.

Complement is only activated at concentrations of phosphorothioate oligodeoxynucleotides that exceed a threshold value of 40–50 μg/ml [76]. Bb levels remained unchanged from control values at plasma concentrations below the threshold. Remarkably, this threshold concentration is similar for three, 20-mer phosphorothioate oligodeoxynucleotides. When concentrations exceeded the threshold values, there was a rapid and marked rise in Bb, indicative of complement activation. Clinical dose regimens have been designed to avoid plasma oligodeoxynucleotide concentrations that exceed 40–50 μg/ml. To this end, the similarities in plasma pharmacokinetics between monkeys and humans have enabled us to design dose regimens with the desired plasma profiles. These threshold concentrations appear to preclude the use of bolus doses in toxicity studies and in clinical trials. However, there may be differences in species sensitivity to complement activation.

Another effect occurring concurrently with administration of phosphorothioate oligodeoxynucleotides is a transient inhibition of clotting times. A number of different phosphorothioate oligodeoxynucleotides alter the clotting cascade as indicated by a concentration-dependent prolongation of activated partial thromboplastin times (aPTT) [27,75,79–81]. In all studies in animals, aPTT was prolonged more than prothrombin times (PT), indicating that the effect was more pronounced in the intrinsic pathway than the extrinsic pathway.

The prolongation of aPTT is highly transient, directly proportional to plasma concentrations of oligodeoxynucleotide, and, therefore, parallels the plasma drug concentration curves with various dose regimens [27,75,79–81]. As drug is cleared from plasma the inhibition diminishes such that there is complete reversal within hours of dosing. With repeated administration, there is no evidence of residual inhibition since prolongations of aPTT are similar on day 1 and day 28 of dosing. Similar observations have been made in clinical studies. The action of phosphorothioate oligodeoxynucleotides on clotting is thought to be the result of the interaction of oligodeoxynucleotides with proteins and in this regard is similar to the activation of complement. Like complement effects, aPTT prolongation is independent of sequence, but dependent on oligonucleotide length.

Phosphorothioate oligonucleotides interfere with the intrinsic pathway at the level of the tenase complex. The site of activity was demonstrated in an elegant series of experiments in which sensitivity to

the prolongation of PTT was modulated by adding in purified clotting factors. Addition of factors distal to the tenase complex did not alter the sensitivity to oligonucleotide-induced prolongation, but adding back factors proximal to the tenase complex regained the inhibitory effects [93].

Phosphorothioate oligonucleotide-induced prolongation of aPTT has been observed in all species examined to date including monkey, rat [27,80,82] and human. Total oligonucleotide concentrations of 10–15 μg/ml in human volunteers were associated with an approx. 50% increase in aPTT [8].

The transient and reversible nature of the aPTT prolongation, combined with the relatively small magnitude of the change, makes these effects clinically insignificant for the current treatment regimens.

Liver is one of the target organs for accumulation of oligonucleotide and is a target organ for toxicity. The immune-mediated cellular infiltrates in rodent livers were discussed above. One hepatocellular change that occurs with administration of oligodeoxynucleotides includes a dose-related hypertrophic change in Kupffer cells accompanied by inclusions of basophilic material. Immunohistochemical analysis of liver using monoclonal antibodies directed toward oligodeoxynucleotides conjugated with KLH demonstrates that these inclusions were composed of oligodeoxynucleotide [83].

At doses in the range of 100–150 mg/kg phosphorothioate oligodeoxynucleotide administered to rodents, there is multi-focal hepatocellular degeneration or single cell necrosis which is usually accompanied by increases in serum transaminases (ALT and AST), and decreased levels of albumin and cholesterol consistent with some degree of mild hepatic dysfunction [4,44,72]. These effects were dose related and doses in the range of 20 mg/kg or below are generally not associated with hepatocellular changes.

In cynomolgus monkeys, 50 mg/kg of ISIS 2302 administered every other day for 4 weeks by intravenous injection produced no morphologic indication of liver toxicity although there was a slight (1.5-fold) increase in AST in this group [72]. Higher doses of other oligonucleotides given s.c. produced Kupffer cell hypertrophy but no indications of cellular necrosis but minor increases in ALT. In clinical trials with ISIS 2302 ISIS 3521 and ISIS 5132 at doses of 2 mg/kg administered by 2 h infusion on alternate day for 3–4 weeks, there was no indication of hepatic dysfunction nor was there any evidence of transaminemia. In contrast, reports on GEM 91 antisense indicate that transaminemias were observed in some AIDS patients treated with repeated administration of the phosphorothioate oligodeoxynucleotide, although these serum enzyme changes were transient and reversed with continued treatment.

The kidney is the site of the highest concentration of oligonucleotide following intravenous injections. These high concentrations are associated with the appearance of basophilic inclusions particularly in the proximal tubule cells that are dose-dependent. The inclusions have been demonstrated to contain oligonucleotide.

In mice, doses of 100 or 150 mg/kg of phosphorothioate oligodeoxynucleotides administered 3 times a week for 2 weeks produced renal proximal tubular degeneration but damage was not sufficient to induce elevations in blood urea nitrogen or creatinine [44]. Higher doses in rats and monkeys (up to 80 mg/kg s.c.) have induced both histologic and serum chemistry changes. Higher doses for more prolonged periods of time can induce functional changes. Dose-response studies demonstrate that in the absence of morphologic changes, there are no changes in other urinary markers of renal function, including β_2-microglobulin, *N*-acetylglucosaminidase (NAG), and retinol binding protein [84,85]. At 10 mg/kg in the monkey, there was no indication of renal dysfunction and the morphologic changes consist of slight variations in brush border or proximal tubule cell heights and active looking nuclei. These changes have been characterized as minimal to mild tubular atrophic and regenerative changes. At a dose of 3 mg/kg and below, these changes were only infrequently observed (if at all). No evidence of renal dysfunction has been observed in clinical trials.

Bone marrow is also a site for accumulation of phosphorothioate oligonucleotides. Heroic doses in mice produce morphologic changes in the bone marrow after 2 weeks of treatment (3 doses/week). There was a reduction in the number of megakaryocytes that was accompanied by approx. 50% reduction in circulating platelet counts [44]. Reductions in platelets have also been observed in the absence of effects on bone marrow. In fact it has been possible to cor-

relate increases in spleen weights with reductions in platelets.

In mice, we have observed that the more highly immunostimulatory oligonucleotides the more profound the change in spleen weight and more profound reductions in platelet number (unpublished observation, Isis Pharmaceuticals). In monkeys, there are transient reductions in platelets that are observed only during the infusion period. These reductions reverse after completion of the infusion. Thrombocytopenia has been observed in AIDS patients treated with GEM 91 [86] and has been reported in cancer patients dosed with phosphorothioate oligonucleotides by constant infusion (unpublished observation, Isis Pharmaceuticals).

3.5. Genotoxicity of phosphorothioate oligonucleotides

Traditional genotoxicity assays have been performed on a number of different phosphorothioate oligonucleotides. Both in vitro and in vivo assays have been performed and to date, in our experience, there have been no positive results. Concentrations in in vitro assays were generally at the proscribed maxima of 5000 μg/ml and in vivo assays were run at 75% of the LD_{50}. The spectrum of studies ranges from the traditional Ames *Salmonella* assay to unscheduled DNA synthesis assays and in vivo micronucleus tests. While not part of traditional genotoxicity assays, we have in recent studies demonstrated that under the conditions of in vitro assay there is uptake of the parent oligonucleotide and its metabolites into the test cells. Furthermore, there is ample evidence in the literature that oligonucleotides are taken up in bone marrow cells. Thus, there is exposure of the test cell lines or cells to the oligonucleotides. These data demonstrate that the negative results obtained in the existing studies were observed under conditions where there was exposure to both parent compound and metabolites.

As for concern about incorporation of phosphorothioate oligonucleotides into genomic DNA, being single stranded they have little chance to incorporate into the genome by recombination. It is theoretically impossible that the RNA-oligodeoxynucleotide complex would participate in homologous recombination and the data collected in genotoxicity assays demonstrate this. This effect is made impossible by the fact that the RNA-oligodeoxynucleotide complex is rapidly degraded by RNase H.

Phosphorothioate oligodeoxynucleotides are composed of endogenous nucleotides and are metabolized by exonucleases to yield thiophosphate mononucleotides that differ from endogenous nucleotides only by the substitution of a sulfur atom for an oxygen atom in the phosphate moiety. The fate of these thiophosphate mononucleotides has not been fully elucidated, but it is possible to infer metabolic pathways from the existing data. From metabolism studies with oligodeoxynucleotide labeled at the C-2 of thymine, we have determined that 50% or more of the nucleotides liberated by the nuclease cleavage of the oligonucleotide are catabolized to CO_2. Assuming the nucleotide follows the normal catabolic route, we can infer that the nucleotide is catabolized by the removal of the thiophosphate leaving the nucleoside. The thiophosphate would then be diluted in the vast compartment of phosphate pools or spontaneously oxidized to phosphate with the liberation of sulfate. The nucleoside could then be a substrate for a nucleoside phosphorylase yielding the ribose sugar and the base that can ultimately be metabolized to CO_2. This pathway suggests that for each $^{14}CO_2$ liberated from the thymine there is the release of a thiophosphate moiety. Because 50% or greater of the radiolabel is detected as CO_2, this suggests that at least half of the molecule including the sulfur is metabolized by normal pathways to yield innocuous inorganic metabolites [1,6]. When a phosphorothioate oligonucleotide is radiolabeled with ^{35}S in the thioate linkage more than half of the radiolabel is detected in urine as low molecular weight metabolites. These data are consistent with the ^{14}C data, suggesting that the ultimate fate of the sulfur is in small molecular weight fractions like thiophosphates, sulfate, or mono- or di-deoxynucleotides.

There are other possible pathways for the thiophosphate mononucleotides that are released by exonuclease cleavage. One pathway would be oxidation of the thiophosphate moiety to phosphate liberating sulfate. We know that oxidation of the phosphorothioate is a common reaction that occurs even under storage conditions. Alternatively, the thiophosphate mononucleotide could be dephosphorylated by the ubiquitous phosphatases, releasing thiophosphate whose fate was described above.

Mononucleotides from the exonuclease cleavage reactions can be phosphorylated and incorporated into nucleotide triphosphate pools. However, it is unlikely that this is a very important pathway considering the sizable fraction of radiolabeled sulfur that is eliminated in the urine of animals treated with ^{35}S-labeled phosphorothioate oligonucleotide. Note that the thiophosphate would still be susceptible to oxidation to the endogenous phosphodiester in all of the phosphorylation states. As part of the triphosphate pool, the nucleotide thiotriphosphate (dNTPαS) could be incorporated in nucleic acids. However, the incorporation is stereospecific and only the S form of the thiotriphosphate is a substrate for the polymerases [87,88]. There are at least two potential consequences of this incorporation. First, the thioate is oxidized and sulfate is released. Second, the molecule is stable and hybridizes in a sequence-dependent manner with the appropriate complementary nucleotide on the opposite strand. Is this important toxicologically? The existing data in genotoxicity assay, cytotoxicity experiments, and acute toxicity studies in vivo all indicate that, if any incorporation occurs, it does not produce genetic alterations. Nor is it producing significant toxicity, since both the cytotoxicity and the acute toxicity of these compounds are low.

4. Reproductive effects of antisense oligodeoxynucleotides

Antisense compounds have shown activity in in vitro models of embryonic development and have been used to study the effects of altering gene transcription on morphogenesis. Sadler [89,90] used antisense oligodeoxynucleotides as a tool to knockout the expression of a specific gene product, Engrailed-1 (*En-1*), in cultured mouse embryos. These experiments demonstrated that sequence-dependent alterations in developing mice embryos could be produced by a single intra-yolk sac injection of 15–25 μM oligodeoxynucleotide. At these concentrations no malformations were observed in embryos treated with control oligodeoxynucleotide. The malformations produced by this direct injection technique were reminiscent of the malformations observed in transgenic mice lacking *En-1*. Yolk sac injections in mouse embryos of an antisense phosphorothioate oligodeoxynucleotides (final concentration 25 μM) directed to retinol binding protein produced apparent reductions in retinol, while a control oligodeoxynucleotide did not [91]. These data suggest that phosphorothioate oligodeoxynucleotides can have activity in embryos when they are exposed directly at high concentrations and at the critical times of gestation. The effects on developing embryos were sequence specific.

When phosphorothioate oligodeoxynucleotides directed toward the developmentally significant gene *Sry* or control oligodeoxynucleotides were administered to pregnant mice at various stages of gestation, at a dose of approx. 18 mg/kg/dose, there were no abnormalities observed [92]. Recent results with ISIS 2302 in rabbits and mice and a mouse-specific anti-ICAM oligodeoxynucleotide, ISIS 3082, in mice, indicate that there were no malformations in either species when given at doses of 12 mg/kg/day during the critical periods of embryogenesis. The data also demonstrate that there were no effects on reproductive performance or fertility in male and female mice at these doses. Note that ISIS 3082 is pharmacologically active in mice and was used to test whether alterations in ICAM-1 expression might have developmental consequences. The only alteration observed was a reduction in fetal weight in rabbits that was associated with a reduction in maternal weight and evidence of slight maternal toxicity. For example, in segment 2 teratogenicity studies with an antisense inhibitor of human papilloma virus (ISIS 2105), there was a low incidence of hydrocephalus in two different litters. The dams of both litters had significant dermal reaction to the intradermal injections of the high dose and the response is regarded as indicative of stress associated with dermal lesions. Taken together these data suggest that at the doses administered there was no evidence of class-related alterations in development or fertility. Whether or not an antisense oligodeoxynucleotide has developmental effects when injected in vivo will need to be assessed for each compound and will be dependent on the target gene, the time of administration, and the pharmacokinetics of oligodeoxynucleotide in the test species. Transplacental kinetics of antisense oligonucleotides need to be characterized. Clearly, there is no

evidence to date suggesting in utero alteration in gene expression by a direct antisense mechanism.

5. Conclusion

There is a growing body of data characterizing the toxicity and pharmacokinetics of antisense oligonucleotides. These data demonstrate that these compounds can be safely administered and that the drugs themselves reach a broad range of target tissues. We continue to add to our understanding of this molecules as we move towards even more potent and more durable antisense compounds.

References

[1] P.A. Cossum, L. Troung, S.R. Owens, P.M. Markham, J.P. Shea, S.T. Crooke, J. Pharmacol. Exp. Ther. 269 (1994) 89–94.
[2] S.T. Crooke, L.R. Grillone, A. Tendolkar, A. Garrett, M.J. Fratkin, J. Leeds, W.H. Barr, Clin. Pharm. Ther. 56 (1994) 641–646.
[3] S.P. Henry, J. Leeds, P.C. Giclas, N.A. Gillett, J.P. Pribble, D.J. Kornbrust, A.A. Levin, Toxicologist 30 (1996) 112.
[4] S.P. Henry, L.R. Grillone, J.L. Orr, R.H. Brunner, D.J. Kornbrust, Toxicology 116 (1997) 77–88.
[5] J.M. Leeds, A. Kiorpes, R.S. Geary, S.P. Henry, C. Goldensoph, A.A. Levin, Pharm. Res. 13 (1996) S–395.
[6] P.A. Cossum, H. Sasmor, D. Dellinger, L. Truong, L. Cummins, S.R. Owens, P.M. Markham, J.P. Shea, S.T. Crooke, J. Pharmacol. Exp. Ther. 267 (1993) 1181–1190.
[7] S. Agrawal, X. Zhang, Z. Lu, H. Zhao, J.M. Tamburin, J. Yan, H. Cai, R.G. Diasio, I. Habus, Z. Jlang, R.P. Iyer, D. Yu, R. Zhang, Biochem. Pharmacol. 50 (1995) 571–576.
[8] J.M. Glover, J.M. Leeds, T.G.K. Mant, D.L. Kisner, J. Zuckerman, A.A. Levin, W.R. Shanahan, J. Pharmacol. Exp. Ther. 282 (1997) 1173–1180.
[9] S.T. Crooke, M.J. Graham, J.E. Zuckerman, D. Brooks, B.S. Conklin, L.L. Cummins, M.J. Greig, C.J. Guinosso, D. Kornbrust, M. Manoharan, H.M. Sasmor, T. Schleich, K.L. Tivel, R.H. Griffey, J. Pharmacol. Exp. Ther. 277 (1996) 923–937.
[10] S. Agrawal, J. Temsamani, J.Y. Tang, Proc. Natl. Acad. Sci.USA 88 (1991) 7595–7599.
[11] P. Iversen, Anti-Cancer Drug Des. 6 (1991) 531–538.
[12] Y. Saijo, L. Perlaky, H. Wang, H. Busch, Oncol. Res. 6 (1994) 243–249.
[13] H. Sands, L.J. Gorey-Feret, A.J. Cocuzza, F.W. Hobbs, D. Chidester, G.L. Trainor, Mol. Pharmacol. 45 (1994) 932–943.
[14] S.K. Srinivasan, P. Iversen, J. Clin. Lab. Anal. 9 (1995) 129–137.
[15] J.M. Leeds, K. Williams, S. Scherrill, A.A. Levin, S. Bistner, S.P. Henry, Fundam. Appl. Toxicol. 30 (1996) 40.
[16] J.M. Leeds, R.S. Geary, in: S.T. Crooke (Ed.), Antisense Research and Applications, vol. 131, 1st edn., Springer, Heidelberg, 1998, pp. 217–231.
[17] M. Deserres, M.J. Mcnulty, L. Christensen, G. Zon, J.W.A. Findlay, Anal. Biochem. 233 (1996) 228–233.
[18] A. Rifai, W. Byrsch, K. Fadden, J. Clark, K.-H. Schlingensipen, Am. J. Pathol. 149 (1996) 717–725.
[19] A.A. Levin, S.P. Henry, C.F. Bennett, D.L. Cole, G.E. Hardee, G.S. Srivatsa, in: D.L. Oxender, L.E. Post (Eds.), Novel Therapeutics from Modern Biotechnology: from Laboratory to Human Testing, vol. 137, 1st edn., Springer-Verlag, Heidelberg, 1998, pp. 131–163.
[20] R.S. Geary, J.M. Leeds, S.P. Henry, D. Monteith, L. Truong, J. Fitchett, T. Burckin, A.A. Levin, in: D.P. Cook (Ed.), Proceedings of the 12th International Roundtable – Nucleosides, Nucleotides and their Biological Applications, La Jolla, CA, 1997, p. 186.
[21] A.A. Levin, R.S. Geary, J.M. Leeds, D.K. Monteith, R. Yu, M.V. Tëmplin, S.P. Henry, in: J.A. Thomas (Ed.), Biotechnology and Safety Assessment, vol. 1, 2nd edn., Taylor and Francis, Philadelphia, PA, 1998, pp. 151–176.
[22] R.S. Geary, J.M. Leeds, J. Fitchett, T. Burckin, L. Troung, C. Spainhour, M. Creek, A.A. Levin, Drug Metab. Dispos. 25 (1997) 1272–1281.
[23] M. Butler, K. Stecker, C.F. Bennett, Lab. Invest. 77 (1997) 379–388.
[24] R. Oberbauer, G.F. Schreiner, T.W. Meyer, Kidney Int. 48 (1995) 1226–1232.
[25] J. Temsamani, J.-Y. Tang, A. Padmapriya, M. Kubert, S. Agrawal, Antisense Res. Dev. 3 (1993) 277–284.
[26] L.L. Cummins, J.M. Leeds, M. Greig, R.J. Griffey, M.J. Graham, R. Crooke, H.J. Gaus, in: IRT: Nucleosides and Nucleotides, vol. 1, La Jolla, CA, 1996, p. 72.
[27] P.L. Nicklin, J. Ambler, A. Mitchelson, D. Bayley, J.A. Phillips, S.J. Craig, B.P. Monia, Proc. Natl. Acad. Sci. USA 16 (1997) 1145–1153.
[28] P. Nicklin, S. Craig, J. Phillips, in: S.T. Crooke (Ed.), Antisense Research and Applications, vol. 131, 1st edn., Springer-Verlag, Berlin, 1998, pp. 141–168.
[29] H.J. Gaus, S.R. Owens, M. Winniman, S. Cooper, L.L. Cummins, Anal. Chem. 69 (1997) 313–319.
[30] R.H. Griffey, M.J. Greig, H.J. Gaus, K. Liu, D. Monteith, M. Winniman, L.L. Cummins, J. Mass Spectrom. 32 (1997) 305–313.
[31] C.F. Bennett, M.-Y. Chiang, L. Wilson-Lingardo, J.R. Wyatt, Nucleic Acids Res. 22 (1994) 3202–3209.
[32] T.L. Burgess, E.F. Fisher, S.L. Ross, J.V. Bready, Y.-X. Qian, L.A. Bayewitch, A.M. Cohen, C.J. Herrera, S.S.-F. Hu, T.B. Kramer, F.D. Lott, F.H. Martin, G.I.F. Pierce, L. Simonet, C.L. Farrell, Proc. Natl. Acad. Sci. USA 92 (1995) 4051–4055.

[33] J.-Y. Maltese, H.W. Sharma, L. Vassilev, R. Narayanan, Nucleic Acids Res. 23 (1995) 1146–1151.
[34] C.A. Stein, Trends Biotechnol. 14 (1996) 147–149.
[35] A.M. Krieg, C.A. Stein, Antisense Res. Dev. 5 (1995) 241.
[36] J.R. Williamson, Curr. Opin. Struct. Biol. 3 (1993) 357–362.
[37] J.R. Wyatt, T.A. Vickers, J.L. Roberson, R.W. Buckheit Jr., T. Klimkait, E. DeBaets, P.W. Davis, B. Rayner, J.L. Imbach, D.J. Ecker, Proc. Natl. Acad. Sci. USA 91 (1994) 1356–1360.
[38] A.M. Krieg, A.-K. Yi, S. Matson, T.J. Waldschmidt, G.A. Bishop, R. Teasdale, G.A. Koretzky, D.M. Klinman, Nature 374 (1995) 546–549.
[39] T. Sparwasser, L. Hultner, E.S. Koch, A. Luz, G.B. Lipford, H. Wagner, J. Immunol. 162 (1999) 2368–2374.
[40] Z.K. Ballas, W.L. Rasmussen, A.M. Krieg, J. Immunol. 157 (1996) 1840–1845.
[41] J.H. Chace, N.A. Hooker, K.L. Mildenstein, A.M. Krieg, J.S. Cowdery, Clin. Immunol. Immunopathol. 84 (1997) 185–193.
[42] D.K. Monteith, S.P. Henry, R.B. Howard, S. Flournoy, A.A. Levin, C.F. Bennett, S.T. Crooke, Anti-Cancer Drug Des. 12 (1997) 421–432.
[43] S.P. Henry, J. Taylor, L. Midgley, A.A. Levin, D.J. Kornbrust, Antisense Nucleic Acid Drug Dev. 7 (1997) 473–481.
[44] U.M. Sarmiento, J.R. Perez, J.M. Becker, N. Ramaswamy, Antisense Res. Dev. 4 (1994) 99–107.
[45] D.M. Klinman, Y. Ae-Kyung, S.L. Beaucage, J. Conover, A.M. Krieg, Proc. Natl. Acad. Sci. USA 93 (1996) 2879–2883.
[46] A.-K. Yi, J.H. Chace, J.S. Cowdery, A.M. Kreig, J. Immunol. 156 (1996) 558–564.
[47] R.M. Crooke, S.T. Crooke, M.J. Graham, M.E. Cooke, Toxicol. Appl. Pharmacol. 140 (1996) 85–93.
[48] R.M. Bennett, G.T. Gabor, M.M. Merritt, Am. Soc. Clin. Invest. 76 (1985) 2182–2190.
[49] A. Krieg, in: S.T. Crooke (Ed.), Antisense Research and Applications, vol. 131, 1st edn., Springer-Verlag, Berlin, 1998, pp. 217–262.
[50] A.M. Krieg, L. Love-Homan, A.K. Yi, J.T. Harty, J. Immunol. 161 (1998) 2428–2434.
[51] A.M. Krieg, A.K. Yi, J. Schorr, H.L. Davis, Trends Microbiol. 6 (1998) 23–27.
[52] D.K. Monteith, A.A. Levin, Toxicol. Pathol. 27 (1999) 8–13.
[53] H.L. Davis, R. Weeranta, T.J. Waldschmidt, L. Tygrett, J. Schorr, A.M. Krieg, J. Immunol. 160 (1998) 870–876.
[54] Z. Moldoveanu, L. Love-Homan, W.Q. Huang, A.M. Krieg, Vaccine 16 (1998) 1216–1224.
[55] G.J. Weiner, H.M. Liu, J.E. Wooldridge, C.E. Dahle, A.M. Krieg, Proc. Natl. Acad. Sci. USA 94 (1997) 10833–10837.
[56] J.E. Wooldridge, Z. Ballas, A.M. Krieg, G.J. Weiner, Blood 89 (1997) 2994–2998.
[57] M.-Y. Chiang, H. Chan, M.A. Zounes, S.M. Freier, W.F. Lima, C.F. Bennett, J. Biol. Chem. 266 (1991) 18162–18171.
[58] C.F. Bennett, Clin. Chem. 40 (1994) 644–645.
[59] N.M. Dean, R. McKay, T.P. Condon, C. Bennett, J. Biol. Chem. 269 (1994) 16416–16424.
[60] S.M. Stepkowski, Y. Tu, T.P. Condon, C.F. Bennett, J. Immunol. 153 (1994) 5336–5346.
[61] C.F. Bennett, N. Dean, D.J. Ecker, B.P. Monia, in: S. Agrawal (Ed.), Methods in Molecular Medicine: Antisense Therapeutics, Humana Press, Totowa, NJ, 1996, pp. 13–46.
[62] L. Miraglia, T. Geiger, C.F. Bennett, N. Dean, Int. J. Immunopharmacol. 18 (1996) 227–240.
[63] C.F. Bennett, D. Kornbrust, S. Henry, K. Stecker, R. Howard, S. Cooper, S. Dutson, W. Hall, H.I. Jacoby, J. Pharmacol. Exp. Ther. 280 (1997) 988–1000.
[64] C.F. Bennett, T. Condon, S. Grimm, H. Chan, M.-Y. Chiang, J. Immunol. 152 (1994) 3530–3540.
[65] T.A. Bacon, E. Wickstrom, Oncogene Res. 6 (1991) 13–19.
[66] J. Goodchild, E. Carroll, J.R. Greenberg, Arch. Biochem. Biophys. 263 (1988) 401–409.
[67] W.F. Lima, V. Brown-Driver, M. Fox, R. Hanecak, T.W. Bruice, J. Biol. Chem. 272 (1997) 626–638.
[68] W.F. Lima, B.P. Monia, D.J. Ecker, S.M. Freier, Biochemistry 31 (1992) 12055–12061.
[69] R.W. Wagner, M.D. Matteucci, D. Grant, T. Huang, B.C. Froehler, Nat. Biotechnol. 14 (1996) 840–844.
[70] D.A. Brown, S.-H. Kang, S.M. Gryaznov, L. DeDionisio, O. Heidenreich, S. Sullivan, X. Xu, M.I. Nerenberg, J. Biol. Chem. 269 (1994) 26801–26805.
[71] S.K. Srinivasan, H.K. Tewary, P.L. Iversen, Antisense Res. Dev. 5 (1995) 131–139.
[72] S.P. Henry, H. Bolte, C. Auletta, D.J. Kornbrust, Toxicology 120 (1997) 145–155.
[73] K.W. McIntyre, K. Lombard-Gilloly, J.R. Perez, C. Kunsch, U.M. Sarmiento, J.D. Larigan, K.T. Landreth, R. Narayanan, Antisense Res. Dev. 3 (1993) 309–322.
[74] W.M. Galbraith, W.C. Hobson, P.C. Giclas, P.J. Schechter, S. Agrawal, Antisense Res. Dev. 4 (1994) 201–206.
[75] T.L. Wallace, S.A. Bazemore, D.J. Kornbrust, P.A. Cossum, J. Pharmacol. Exp. Ther. 278 (1996) 1306–1312.
[76] S.P. Henry, P.C. Giclas, J. Leeds, M. Pangburn, C. Auletta, A.A. Levin, D.J. Kornbrust, J. Pharmacol. Exp. Ther. 281 (1997) 810–816.
[77] K.G. Cornish, P. Iversen, L. Smith, M. Arneson, E. Bayever, Pharmacol. Commun. 3 (1993) 239–247.
[78] D. Bitter-Suermann, R. Burger, U. Hadding, Eur. J. Immunol. 11 (1981) 291–295.
[79] L.C. Griffin, G.F. Tidmarsh, L.C. Bock, J.J. Toole, L.L.K. Leung, Blood 81 (1993) 3271–3276.
[80] S.P. Henry, W. Novotny, J. Leeds, C. Auletta, D.J. Kornbrust, Antisense Nucleic Acid Drug Dev. 7 (1997) 503–510.
[81] S.P. Henry, R. Larkin, W.F. Novotny, D.J. Kornbrust, Pharm. Res. 11 (1994) S-353.
[82] S.P. Henry, D. Monteith, A.A. Levin, Anti-Cancer Drug Des. 12 (1997) 395–408.
[83] M. Butler, K. Stecker, C.F. Bennett, Nucleosides Nucleotides 16 (1997) 1761–1764.
[84] M.J. Horner, D.K. Monteith, N.A. Gillett, M. Butler, S.P. Henry, C.F. Bennett, A.A. Levin, Fundam. Appl. Toxicol. 36 (1997) 147.
[85] L.L. Manza, M.M. Butler, C.F. Bennett, A.A. Levin, A.A.

de Peyster, D.K. Monteith, Fundam. Appl. Toxicol. 36 (1997) 147.
[86] F. Plenat, Mol. Med. Today 2 (1996) 250–257.
[87] F. Eckstein, H.H. Schulz, H. Ruterjans, W. Haar, W. Maurer, Biochemistry 11 (1972) 3507–3512.
[88] F. Eckstein, J.B. Thomson, Methods Enzymol. 262 (1995) 189–203.
[89] K.A. Augustine, E.T. Liu, T.W. Sadler, Teratology 51 (1995) 300–310.
[90] T.W. Sadler, E.T. Liu, K.A. Augustine, Teratology 51 (1995) 292–299.
[91] C. Bavik, S.J. Ward, P. Chambon, Proc. Natl. Acad. Sci. USA 93 (1996) 3110–3114.
[92] M.F. Gaudette, G. Hampikian, V. Metelev, S. Agrawal, W.R. Crain, Antisense Res. Dev. 3 (1993) 391–397.
[93] J.P. Sheehan, L. Hao-Chang, Blood 92 (1998) 1617–1625.

Targeting DNA with Triplexes

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Abstract: The formation of intermolecular DNA triple helices offers the possibility of designing compounds with extensive sequence recognition properties which may be useful as antigene agents or tools in molecular biology. In these structures a third strand oligonucleotide binds in the DNA major groove, making specific contacts with substituents on the exposed faces of the base pairs. Although triplexes form with exquisite specificity their use suffers from several drawbacks. Two limitations of this approach, which are considered in this review are, firstly that conditions of low pH are necessary for formation of the C^+•GC triplet, and secondly that these structures are often less stable than their duplex counterparts. This review outlines the strategies that have been employed to overcome these drawbacks. The pH problem is addressed by considering the various DNA base analogues that have been used to recognise GC base pairs in a pH independent fashion, and discusses the benefits and limitations of each analogue. Triplex stability can be increased by using novel base analogues, backbone modifications and the use of triplex-specific binding ligands.

Background

Compounds which interact with duplex DNA in a sequence-specific fashion have the potential to inhibit the activity of individual genes, and may be useful for treating a variety of diseases including cancer or viral infections. In principle it should be simpler to design drugs targeted to DNA than against many other pharmacological receptors, since the structure of the target is well known in precise molecular detail. In addition there are only two copies of each DNA target site per diploid cell, and it may therefore be possible to use low doses of such agents, provided that they maintain high affinity and stringency. In order to be selective for a unique DNA sequence within the human genome of 3×10^9 base pairs, such agents need to recognise at least 16-17 consecutive bases. Sequence specific recognition of DNA is achieved by making specific contacts with the regular array of hydrogen bond donors and acceptors on the base pairs which are exposed in the major and minor grooves. Since these recognition elements are positioned in a helical arrangement, turning through about 360° every 10 base pairs, DNA sequence-reading agents need to follow this helical pattern. A versatile code for generating sequence-specific DNA-reading agents will therefore require monomer units which selectively bind to the individual bases (or base pairs), and which are attached to a regular backbone which is able to wind around the DNA helix, possessing the same axial rise as duplex DNA. A simple solution to these problems is to use oligonucleotides as sequence specific agents, since their repeating unit is the same as that of duplex DNA, and they can easily adopt a helical structure. It has been known for several years that the major groove of duplex DNA is large enough to accommodate a third oligonucleotide strand, forming an intermolecular triplex. By exploring the rules governing the formation of these complexes it is hoped to generate a versatile recognition code for designing agents to interact with any desired sequence.

The formation of DNA and RNA triple helices was first demonstrated in 1957 by mixing polyU and polyA in the ratio of 2:1 [1,2]. This and other triplexes, formed with synthetic polynucleotides, remained an obscure part of DNA chemistry until 1987 when it was realised that they offered a means for designing DNA sequence specific agents [3,4]. Since then a variety of biological activities have been proposed for these structures [5-10]. In these complexes the third strand lies in the major groove of the target DNA duplex, where it makes specific hydrogen bond contacts with substituents on the exposed faces of the duplex DNA base pairs. Two types of triplexes have been characterised, which differ in the orientation of the third strand. Those in which the third strand runs parallel to the duplex purine strand are characterised by T•AT and C^+•GC triplets, Fig. (**1a**),

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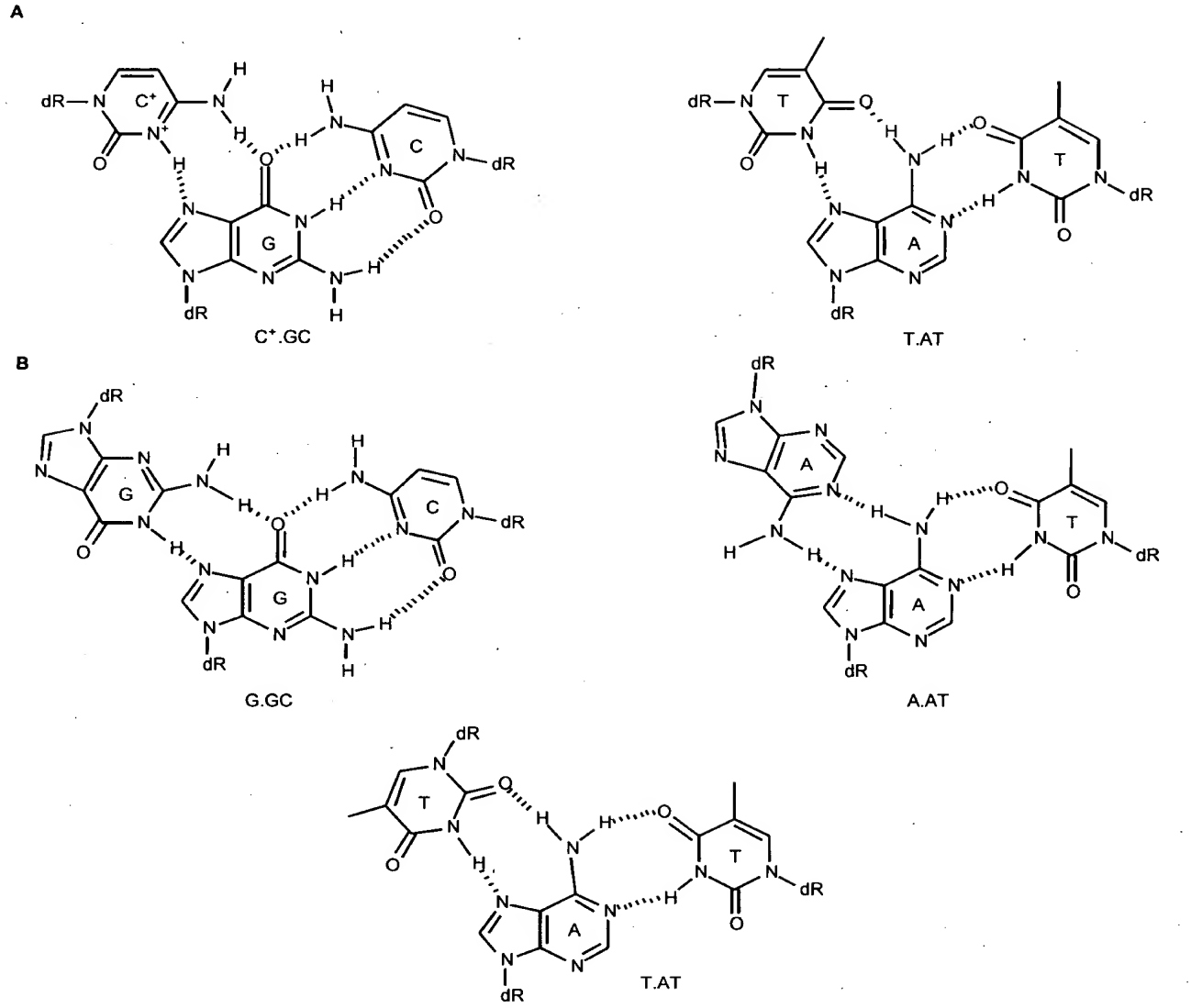

Fig. (1). A) Structures of the parallel triplets C$^+$•GC and T•AT. B) Structures of the antiparallel triplets G•GC, A•AT and T•AT.

and are stabilised by the formation of Hoogsteen base pairs [3,4,11]. Within this motif the C$^+$•GC triplet requires conditions of low pH (<6.0), necessary for protonation of the third strand cytosine. In contrast antiparallel triplexes are characterised by A•AT, T•AT and G•GC triplets, Fig. (**1b**) and are stabilised by reverse-Hoogsteen base pairs [12- 14]. [In the following, the notation X•ZY denotes a triplet in which the third strand base X interacts with a ZY base pair forming hydrogen bonds to base Z]. The details of the structures and applications of these standard triplexes have been considered in several recent reviews [4-10].

Although intermolecular triple helix formation offers the possibility of selectively targeting unique DNA sequences, it currently suffers from several limitations, two of which are considered in this review. Firstly, parallel triplex formation requires conditions of low pH necessary for formation of the C$^+$ •GC triplet. Secondly, triplexes form with lower stability than their duplex counterparts. Triplex formation is also generally restricted to homopurine-tracts, and this problem has been considered in recent reviews [15,16].

Cytosine Protonation

Parallel triplex formation generally requires conditions of low pH (< 6.0) necessary for protonation of N3 of the third strand cytosine in the C$^+$•GC triplet. Several cytosine analogues have therefore been designed with the aim of forming Hoogsteen hydrogen

bonds with guanine at physiological pHs. However, before describing these derivatives, it is first worth considering some of the essential features of the C^+•GC triplet, some of which are sometimes overlooked when designing new base analogues.

Firstly, although cytosine protonation requires conditions of low pH, and contiguous C^+•GC triplets are destabilising [17], several studies have shown that C^+•GC imparts a greater triplex stability than T•AT [18-21]. In contrast the unprotonated C•GC triplet, containing only one Hoogsteen hydrogen bond, is less stable than T•AT. The very large stabilisation from protonation is too large to be accounted for by the single additional hydrogen bond [19], and must therefore include contributions from either electrostatic interactions with the phosphate backbone or altered stacking with the neighbouring bases, possibly as a result of favourable interactions between the positive charge and the π-stack. Although the pK of free cytosine is about 4.5, this is elevated on triplex formation and may be as high as 9.0 for an isolated internal cytosine [19]. Terminal C^+•GC triplets are less stable, with lower pKs, presumably because the protonated base is exposed to solvent, and the interaction with only one nearest neighbour is less stabilising than when it can interact with two neighbours [19]. A consequence of the stronger binding of C^+•GC than T•AT is that N3-protonated cytosine analogues which retain the positive charge are likely to produce more stable triplexes than those in which the charge is missing. Additionally it may in future be worth considering the synthesis of charged thymine analogues so as to increase the stability of the T•AT triplet.

A second important factor is that, unlike the different triplet combinations in antiparallel triplexes, C^+•GC and T•AT triplets are isostructural [6, 22]. As a consequence there are no differences in backbone distortion between the various third strand base steps, i.e. TpC, CpT, TpT and CpC, and the base overlap for different steps is also constant. An inevitable consequence of using novel third strand cytosine analogues is that the X•GC triplet may no longer be compatible with T•AT. This may be especially relevant for analogues which are based on a purine rather than a pyrimidine ring. This disadvantage could of course be overcome by simultaneously introducing similarly modified thymine analogues generating X•AT triplets which are isostructural with X•GC.

The free cytosine nucleoside has a pK of about 4.5, though this is elevated at isolated cytosines within triplex forming oligonucleotides, depending on their number and location. Several base analogues have been synthesised in attempts to overcome this restriction, some of which are presented in Figs. (**2**) and (**3**). It can be seen that recognition of guanine requires a structure presenting two hydrogen bond

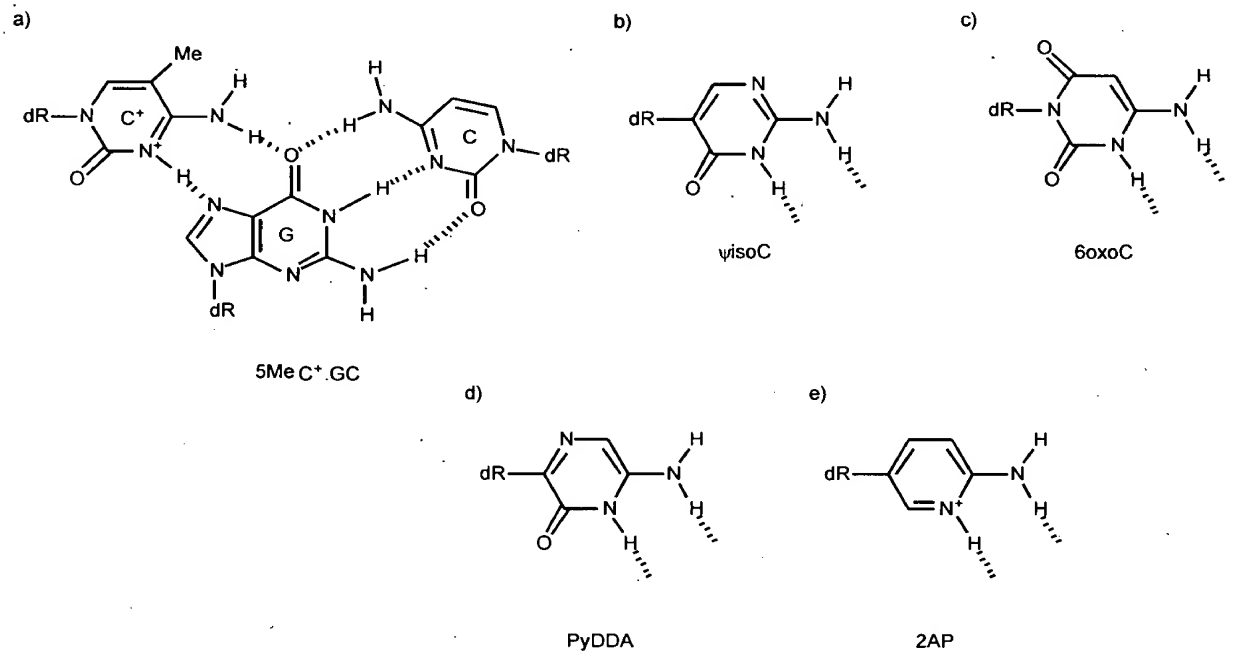

Fig. (2). Pyrimidine analogues for recognition of GC base pairs in a parallel triplex. a) the $^{5Me}C^+$•GC triplet, b) pseudo-isocytosine [27,28], c) 6-oxocytosine [31-34], d) pyrazine analogue [37], e) 2-aminopyridine [38-41]. In b) - e) the third strand base analogue is shown alone in the same orientation as in a), indicating the substituents that form hydrogen bonds to G.

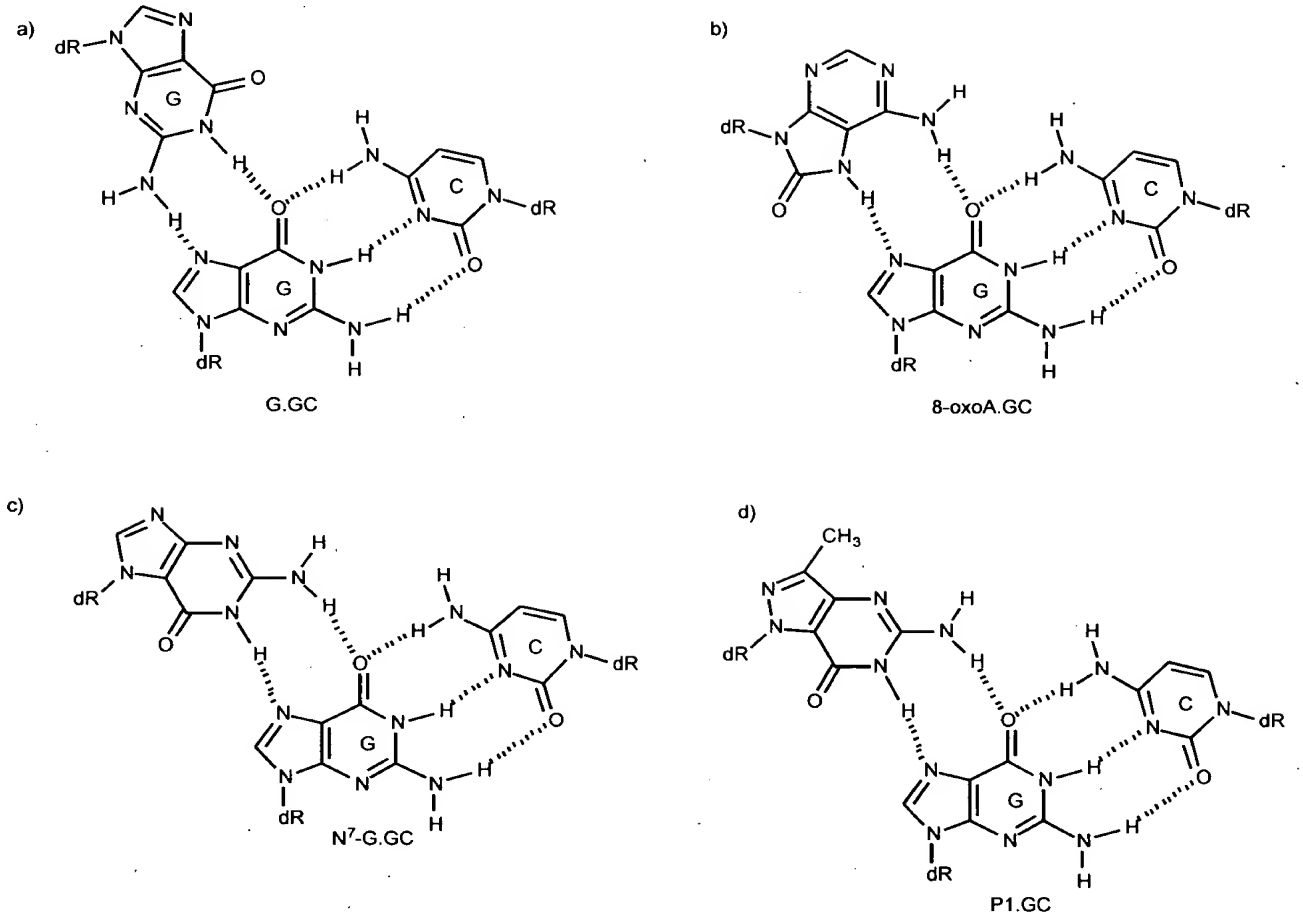

Fig. (3). Purine analogues for recognition of GC base pairs in a parallel triplex. a) the parallel G•GC triplet [20, 44], b) 8-oxoadenine•GC triplet [46-48], c) N^7-G•GC triplet, d) P1•GC triplet.

donors to interact with O6 and N7. These analogues can be divided into two classes which are based on either a pyrimidine or a purine ring structure.

Pyrimidine Analogues

The first cytosine analogue to be tested was 5-methylcytosine, Fig. (**2a**) a naturally occurring base, since this has a higher pK value than cytosine [17, 23, 24]. Triplexes containing this base are indeed more stable at slightly higher pHs, but are still not formed under physiological conditions [25]. The improvement in the pH profile of ^{5Me}C relative to C is better than would be expected from the small change in pK, and it has been suggested that the increased stability results from the extra spine of methyl groups within the DNA major groove. These methyl groups increase stability by either inducing the release of water molecules, thereby contributing a positive entropy change [23] or by improving the base stacking [26]. This improvement in pH profile is accompanied by an increase in affinity [24].

Pseudoisocytosine, Fig. (**2b**) [27,28] has been successfully employed, as its 2'-O- methyl derivative, for triplex recognition of guanine. In the tautomeric form shown in Fig. (**2b**) this base contains a hydrogen at position N3 and has the same potential for forming Hoogsteen hydrogen bonds with G as protonated cytosine. In the alternative tautomer N1 is protonated rather than N3 and it can form a stable base pair with guanine [28].This analogue has been shown to form pH-independent triplets with GC base pairs which are more stable than those formed with natural bases [28]. The formation of C^+•GC triplets is especially difficult in runs of contiguous GC base pairs, presumably because of unfavourable charge interactions between adjacent protonated cytosines, which further decreases their effective pK. Pseudoisocytosine, alone amongst the uncharged pyrimidine analogues (see below) appears to overcome this limitation and has been shown to form

a stable triplex at a site containing six contiguous guanines. Surprisingly this base has not found widespread use in triplex strategies, because of its limited availability and difficult synthesis, but it is more frequently employed as a cytosine analogue in PNA-containing structures [29, 30].

6-Oxocytosine, Fig. (**2c**) is a further pyrimidine analogue which presents the correct arrangement of hydrogen bonds for pH independent recognition of guanine [31-34]. Although this base forms a pH independent triplet with GC, the complexes generated are less stable than those observed with C or ^{5Me}C at low pH. This could reflect the importance of the positive charge noted above, affecting the energetics of base stacking, or might be due to changes in the hydrogen bonding characteristics since the natural bases form one hydrogen bond with a charged partner in contrast to the uncharged partner with the synthetic base. The less efficient base stacking observed with this analogue is consistent with the observation that this base does not form stable triplexes at blocks of contiguous guanines, even though it does not suffer from the charge repulsion seen with C^+ and $^{5Me}C^+$ [33]. Surprisingly stable recognition of G-tracts is achieved by alternating this base analogue with ^{5Me}C [33]. More recently it has been shown that 6-oxocytosine forms a more stable triplet when deoxyribose is replaced with a flexible acyclic linker [34]. In contrast this flexible linker lowered the stability of T•AT triplets. It seems to be a common feature that T•AT triplets are stabilized by rigid linkers while C^+•GC requires a less rigid linker [35, 36]. This most likely reflects the different optimal sugar conformations and base stacking environments required for C^+•GC and T•AT. With 6- oxocytosine triplex stability decreased slightly as the number of acyclic linkers in the oligonucleotide was increased from 2 to 6, presumably because unfavourable entropy effects overcome the enhanced base stacking.

A further uncharged cytosine analogue for GC recognition is based on a pyrazine ring [37] and is shown in Fig. (**2d**). Although there is little information on this analogue it showed pH independent recognition of a single guanine embedded in an AT tract (as its 2'-O-methyl riboside).

Another promising cytosine analogue is 2-aminopyridine (2AP), Fig. (**2e**) which has a pK of 6.86, closer to physiological pH [38-41]. Psoralen-linked oligonucleotides containing this base have been successfully targeted against a portion of the aromatase gene [38]. This analogue forms stable triplexes at higher pHs than either C or ^{5Me}C [39]. This increased stability is also evident at low pHs suggesting that the 2AP•GC triplet is intrinsically more stable. This base analogue also forms stable complexes at blocks of contiguous GC base pairs. In footprinting experiments $(2AP)_6T_6$ formed a triplex with G_6A_6•T_6C_6 at pHs as high as 7.0, in contrast to $^{5Me}C_6T_6$ and C_6T_6, for which complexes are barely stable at pH 5.0 [39]. Surprisingly addition of a 5-methyl group to this base analogue does not affect its activity, and the 2'-O-methyl derivative is less active [41].

An alternative possibility for recognition of GC base pairs at physiological pHs is to retain the use of third strand cytosines, generating a triplet which is stabilized by only one hydrogen bond [42]. Since T•AT is isomorphous with C•GC, the third strand stacking interactions and phosphodiester backbone configurations may allow stable complex formation without the need for two hydrogen bonds in every C•GC triplet.

An alternative approach to base modification for altering the pK of cytosine is to modify the sugar. The pK of the carbocyclic derivative of ^{5Me}C is increased by 0.45 relative to ^{5Me}C and forms more stable triplets [43]. In contrast the carbocyclic analogue of T forms less stable T•AT triplets. This difference may reflect the different preferred sugar conformations adopted by C•GC and T•AT, since the carbocyclic sugar adopts a C1'-*exo* configuration.

Purine Analogues

A different strategy for GC recognition uses purine analogues to position the required hydrogen bond donors and several of these are shown in Fig. (**3**). It should however be noted that these analogues form triplets which are not isostructural with T•AT since the backbone must be in a different position. As a consequence they may need to be used in combination with equivalent analogues of T for recognition of AT pairs. Purine-rich oligonucleotides generally bind to duplex DNA in an antiparallel orientation generating G•GC, A•AT and (reverse Hoogsteen) T•AT triplets. However, it has been shown that GT-containing oligonucleotides can bind in a parallel configuration, Fig. (**3a**) though the strand orientation depends on the number of GpT and TpG steps [22, 44]. Similarly 2-amino-purine has been suggested for recognizing AT pairs in the parallel motif [45].

The first synthetic purine derivatives to be described were 8-oxoadenine [46, 47], Fig. (**3b**) and its N6-methyl analogue [48], which, in the *syn* conformation, are arranged so that the 6-amino and N7 protons replace the 4-amino and N3 protons of cytosine. These analogues have been shown to recognise guanine in a pH independent fashion forming triplets which are as stable as C^+•GC at low pH. 8-oxoA has been shown to form a stable triplex at a site

containing four contiguous guanines, which cannot be targeted with cytosine-containing oligonucleotides. Since this base has been tested in oligonucleotides containing T for recognition of AT pairs, it is clear that the third strand backbone must be distorted at each GA and AG step in the target. A more even backbone might be achieved by using this base in combination with 8-oxoguanine, which in the *syn* conformation, should be able to form Hoogsteen hydrogen bonds to A.

Other purine analogues, N7-G, Fig. (**3c**) [49-51], and P1, Fig. (**3d**) [52-54] bind in an *anti* configuration recognizing GC in a pH independent fashion. Although isolated P1.GC and N7-G.GC triplets show a similar stability to $^{5Me}C^{+}$•GC, recognition of alternating GA sequences by alternating P1 and T or N7-G and T is about three orders of magnitude lower than with oligonucleotides containing ^{5Me}C and T. In contrast, tracts of six contiguous guanines are bound by P1 or N7-G about four orders of magnitude better than ^{5Me}C. These differences are probably caused by the lack of structural isomorphism between N7-G•GC and T•AT or P1•GC and T•AT, whereas no such structural distortion is present within tracts of identical triplets. A similar effect is seen when N7-G is connected by a flexible acyclic linker [55]. The location of the phosphodiester backbone is seen to be important since on moving the glycosidic linkage in P1 from N7 to N8, generating base P2, no triplex formation was observed. These base analogues for GC recognition suggest that it may be possible to generate a new parallel stranded motif based on N7 purines.

A further analogue suggested for recognition of GC base pairs within the parallel motif is N7-inosine. It has been suggested that this base, which can be used for pH independent recognition of GC in both alternating GA and oligo G-tracts, binds by forming a single hydrogen bond between N1H of inosine and guanine N7. Interaction with other base pairs is prohibited by unfavourable repulsive Van der Waal's interaction [56].

Strength Of Binding

Although triplex-forming oligonucleotides bind with high specificity, their binding may not be strong and weaker than that of the underlying DNA duplex. In large part this is thought to be due to the charge repulsion resulting from bringing together the three polyanionic DNA strands. Several strategies, described below, have therefore been adopted for increasing triplex stability.

Positive Charges

One obvious approach for neutralising the charge repulsion between the three negatively charged phosphodiester backbones is to modify either the

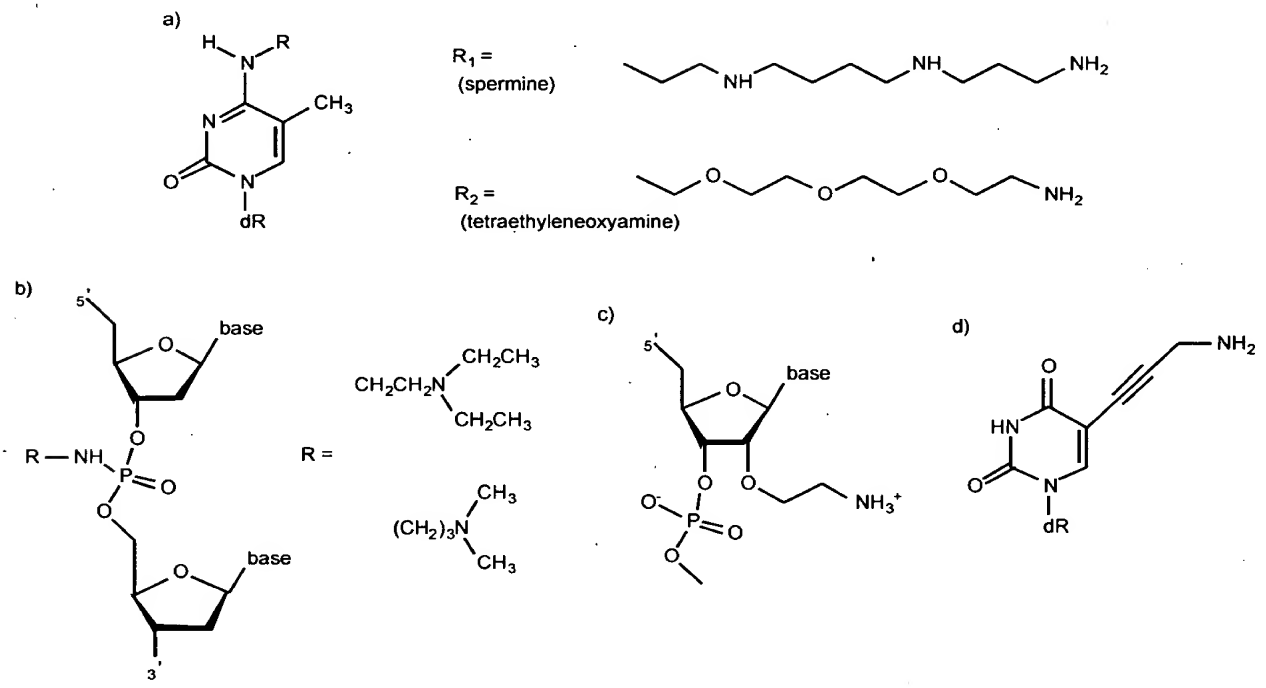

Fig. (4). Positively charged DNA analogues. a) N4-spermine derivatives of methylcytosine [59-62], b) cationic phosphoramidate backbone [67-69], c) 2'-aminoethoxy [70, 71], d) propargylamino-dU [72].

sugar-phosphate backbone or the bases so as to incorporate positive charges. Since triplexes are known to be stabilised by spermine [57, 58] several groups have covalently attached this group to N4 [59-62] of methylcytosine, Fig. (**4a**). Surprisingly these triplexes were stable at pH 7.4, even though N3 should be unprotonated at this pH, suggesting that the lack of the second hydrogen bond in the C^+•GC triplet can be compensated by favourable electrostatic interactions. Indeed these triplexes were less stable at lower pHs. This substitution was more effective when placed at either end of the triplex than at the centre and caused a slight decrease in Tm with increasing number of substitutions. In addition these triplexes were less dependent on the presence of divalent metal ions such as Mg^{2+}. Replacing the polycationic spermine with tetraethoxyleneoxyamine, Fig. (**4a**) produced a similar effect on triplex stability [61], presumably by virtue of favourable hydrophobic, rather than purely electrostatic interactions. Other studies with *syn*-norspermidine linked to the 5-position of U in the third strand also show significant triplex stabilization at physiological pH [63]. Spermine has also been attached to the 5'-terminus [64] and to the 2'-O of oligonucleotides [65, 66]. Both these modifications increase triplex melting temperatures, though 2'-O-linked spermine induced triplex formation when added at the 3'- or 5' end, but not at the centre of the third strand oligonucleotide.

Several studies have examined triplex formation by oligonucleotides containing neutral or positively charged backbones in attempts to alleviate the charge repulsion between the polyanionic strands. Examples of this include the cationic phosphoramidates shown in Fig. (**4b**). These have been shown to stabilise both parallel [67] and antiparallel triplexes [68, 69]. The role of the positive charge is confirmed by studies with an uncharged analogue which showed only a small increase in stability relative to the negatively charged phosphodiester backbone [68]. The triplex binding of such GA-containing phosphoramidates was maintained in millimolar concentrations of potassium, conditions that usually inhibit antiparallel triplex formation as a result of competing structures adopted by the third strand oligonucleotide. These modifications introduce a chiral phosphate, and experiments with stereouniform phosphoramidates have shown that, although one stereoisomer binds with higher affinity than the simple phosphodiesters, the other binds with lower affinity [68].

Other studies have included a positive charge into the backbone by attaching an aminoethoxy moiety to the 2'-position, Fig. (**4c**) [70, 71].This modification causes a dramatic increase in affinity and association rate. Extension of the side chain by an additional methylene group, using a 2'-aminopropoxy group, caused a significant decrease in triplex stability, suggesting that the protonated group is ideally positioned to interact with a nearby phosphate group [70]. Indeed an NMR structure of an intramolecular triplex containing this modification in the Hoogsteen strand has shown contacts between the amino protons of the aminoethoxy group and the phosphates of the purine strand of the duplex [71].

We have also shown that propargylamino-dU, Fig. (**4d**) in which a positively charged group is added to the 5'-position of thymine causes a dramatic increase in triplex stability [72]. The degree of stabilization was pH dependent, confirming the importance of the positive charge. In this case the stabilization is due to more than inclusion of the positive charge since propylamino-dU had a much lower effect on stability.

Improved Base Stacking

The observation that the increased stability of $^{5Me}C^+$•GC relative to C^+•GC is caused by the extra spine of methyl groups within the DNA major groove leads to the suggestion that other analogues with improved base stacking might form more stable triplexes. The methyl group at the 5-position of thymine is also known to contribute to the stability of the T•AT triplet and it has been shown that the ranking order for 5-substituted derivatives is $^{Br}U > T > U$, suggesting that a bulky substituent can improve activity [24]. A similar effect is seen with 5-propynyl-U, Fig. (**5a**), which also increases triplet stability [73-75], though 5-propynyl-C produces a less stable triplet since this substitution further reduces the pK of N3 to 3.3. The increased stability of 5-propynyl substituted bases is presumably due to increased base stacking interactions.

Other studies have attempted to improve base stacking by adding a extra aromatic rings to thymine. One of the first such analogues to be tested was a pyrido[2,3-*d*]pyrimidine derivative (F) [76]. This base selectively recognises AT base pairs, forming the F•AT triplet, utilising the tautomer shown in Fig. (**5b**). However, these complexes were no more stable than those containing T•AT triplets. In addition experiments examining the cooperative binding of adjacent oligonucleotides showed that stacking of F on F was less favourable than T on T. Similarly quinazoline-2,4(1*H*,3*H*)-dione [77, 78], Fig (**5c**), benzo[*f*]quinazoline-2,4-(1*H*,3*H*)-dione, Fig. (**5d**) [79] and benzo[*g*]quinazoline-2,4-(1*H*,3*H*)-dione Fig. (**5e**) [79] have been examined as thymine analogues. Although each of these analogues selectively recognized AT base pairs, in both parallel and antiparallel triplexes, the complexes were less stable than those containing standard T•AT triplets. Taken together these results suggest that modification of pyrimidine bases to form extended ring systems with

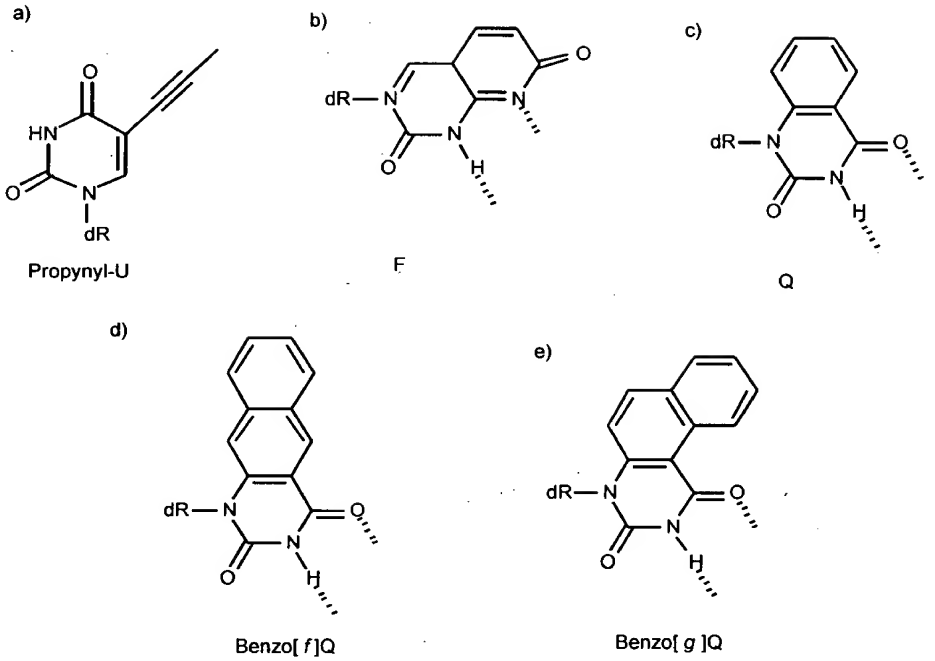

Fig. (5). Base analogues designed to improve third strand base stacking. a) propynyl-dU [73-75], b) pyrido[2,3-*d*]pyrimidine derivative (F) [76], c) quinazoline-2,4(1*H*,3*H*)-dione [77, 78], d) benzo[*f*]quinazoline-2,4-(1*H*,3*H*)-dione, [79], e) benzo[*g*]quinazoline-2,4-(1*H*,3*H*)-dione [79].

the object of increasing the base stacking interactions does not necessarily produce more stable triplex formation.This could either be because the structural parameters of adjacent base triplets do not allow favourable overlap of the additional aromatic rings, or because the increased third strand base stacking disrupts the normal helical parameters, leading to less stable complexes.

Backbone Modifications

RNA

Most triplex studies have examined the formation of triple helices in which all three strands contain DNA, or in which two pyrimidine-containing DNA strands bind to a single stranded RNA (oligo-clamp). There have been a fewer studies on other triplexes containing various combinations of RNA and DNA strands [80-84]. Antiparallel (R•RY) triplexes cannot form if any strand is RNA [80]. In contrast parallel triplexes can be formed with various combinations, though a DNA third strand does not bind against a duplex containing RNA in the purine strand [82, 84]. In general DNA third strands are best for recognizing duplexes with DNA in the purine strand. However there are very great differences in the measured stabilities of various RNA and DNA containing triplexes in different studies. These large discrepancies have been explained by suggesting that the binding affinities of R or D third strands to double helical nucleic acids are length, sequence composition, pH and salt dependent [82]. On the basis of affinity cleavage patterns it has been suggested that Y•RY triplexes fall into two distinct conformational classes [83] dependent on the nature of the backbone i.e. dY•dRdY, rY•dRdY, dY•dRrY and rY•dRrY, rY•rRdY, rY•rRrY.

Inversion of the stereochemistry at the 2'-OH, generating pyrimidine-containing arabinonucleic acid, permits triplex formation only at duplex targets for which the purine strand is DNA (not RNA), in contrast to an RNA containing third strand which formed triplexes at both DNA, RNA and DNA/RNA hybrids under these conditions [85].

2'-OMe

2'-O-methyl-containing oligonucleotides, Fig. (**6b**) are attractive agents for triplex formation since they generally produce more stable parallel triplexes than their RNA or DNA counterparts [86-89] and addition of the 2'-O-methyl group confers resistance against cellular nucleases. Irrespective of the duplex combination (DNA or RNA) the 2'-O-methyl group forms a more stable parallel triplex than a DNA-containing third strand [89].

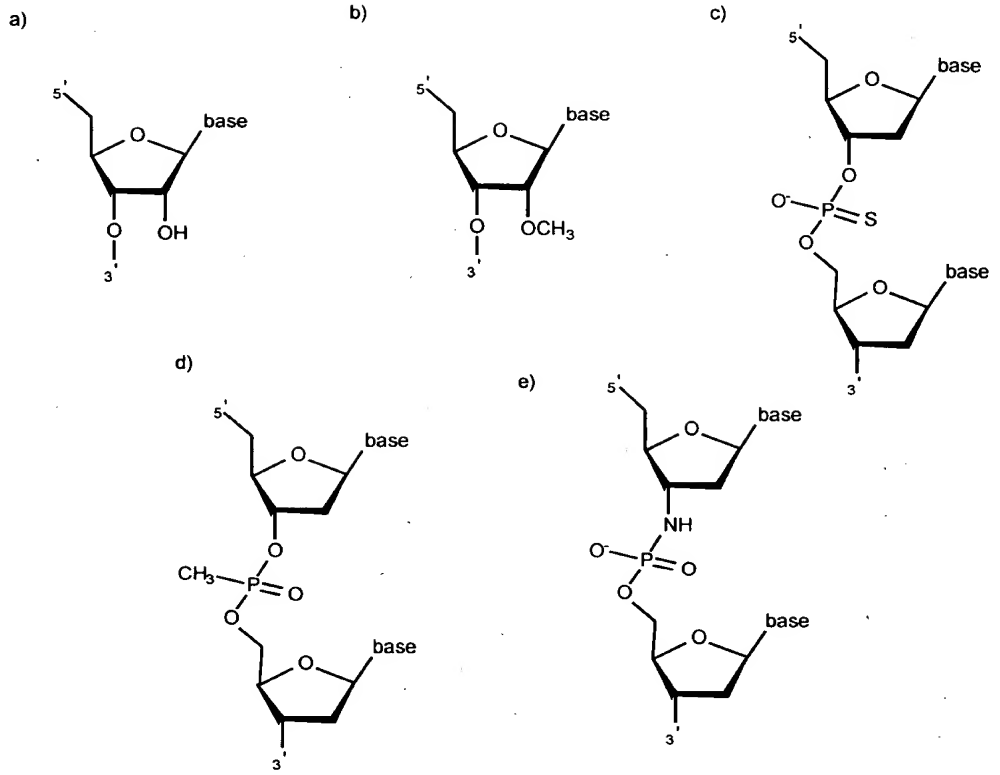

Fig. (6). Modified DNA sugars and backbones for improving triplex stability. a) RNA, b) 2'-OMe-RNA, c) phosphorothioate, d) methylphosphonate, e)N3'→P5' phosphoramidate.

Phosphorothioates

Phosphorothioates, Fig. (**6c**) are promising candidates for oligonucleotide backbone modifications since they possess a similar charge density to normal oligonucleotides, are soluble in aqueous solutions and show reduced degradation by many nucleases. The presence of this group has a dramatic and specific effect on triplex formation. With the phosphorothioate on the purine strand poly(dA-dG).poly(dC-dT) disproportionates into a triplex and single strand, the stability of which increases at lower pHs, while placing the phosphorothioate on the pyrimidine-containing strand inhibits triplex formation [90]. Purine-rich oligonucleotides containing a diastereoisomeric mixture or stereoregular (R_p) phosphorothioate linkages have been shown to form triplexes with similar affinities to phosphodiester linkages oligonucleotides, in contrast to pyrimidine-rich oligonucleotides for which neither diastereomeric or stereoregular (R_p) phosphorothioates formed stable triplexes [91]. GA-containing phosphorothioates form less stable antiparallel triplexes than their phosphodiester counterparts, while a 23-mer GT-containing phosphorothioate did not support triplex formation [92]. For GA-containing third strands it has been shown that inclusion of only two phosphorothioate linkages at each end of a 23-mer oligonucleotide did not reduce the binding affinity, yet was more resistant to nucleases than the phosphodiester oligomer [92]. Antiparallel GA-containing phosphorothioate oligonucleotides have also been shown to inhibit gene transcription *in vivo*. Despite the observation that pyrimidine phosphorothioates do not form parallel triplexes these oligonucleotides have been reported to inhibit T7 polymerase transcription *in vitro* [93].

Methylphosphonates

Nonionic methylphosphonate substitutions, Fig. (**6d**) might be expected to be good candidates for triplex formation since they should overcome the charge repulsion of the third strand in addition to being resistant to nuclease attack. However, there have been conflicting results with oligonucleotides containing this modified backbone. Kibler-Herzog [94] found that no triplexes were produced with dA.dT mixtures when any of the strands consisted of methylphosphonates, though complexes of lower stability could be produced with strands containing alternating phosphodiesters and methylphosphonates [95]. In contrast it has been reported that the formation of a 2:1 complex of $(CT)_8$

with $(AG)_8$ was similar with methylphosphonate and phosphodiester backbones [96]. In addition purine-rich triplex-forming methylphosphonate oligonucleotides have been used to target mRNA [97]. It has also been shown that 2:1 complexes between methylphosphonate dA and phosphodiester dT or methylphosphonate $d(AG)_8$ and phosphodiester $d(CT)_8$ are more stable than those with phosphodiester third strands [98] for which natural $d(AG)_8.d(AG)_8.d(CT)_8$ could not be observed. These complexes also required lower concentrations of cations than normally needed for triplex formation [98]. The affinity of methylphosphonate oligonucleotides may be further increased by inversion of the anomeric configuration from to [99]. In each of these instances the methylphosphonates contained mixtures of R and S-diastereoisomers at the chiral phosphate centre. It remains to be seen whether stereochemically pure methylphosphonates will have better understood triplex forming properties.

N3'→P5' Phosphoramidates

N3'→P5' phosphoramidates, Fig (**6e**) in which O3' is replaced by NH, represent a further modification of the phosphodiester backbone which has been examined for triplex formation. Parallel triplexes containing this modification in the third strand are much more stable than those formed with oligonucleotides with a natural phosphodiester backbone [100,101]. This modification permited stable triplex formation at a target site containing six adjacent guanines at pH 7.0, and was more effective than either ^{5Me}C or tethering an acridine to the 5'-end [100]. By combining the N3'→P5' phosphoramidate backbone with ^{5Me}C the complexes were further stabilized. Phosphoramidate-containing oligonucleotides have been shown to inhibit transcription at pH 7.0 *in vitro*, under conditions in which the natural phosphodiesters have no effect [102]. The avidity of these modified oligonucleotides for forming duplexes and triplexes is such that phosphoramidate dT_{15} partially displaces the dT_{15} stand from a $dT_{15}.dA_{15}$ duplex.[101]. The stability may be further increased by using RNA phosphoramidate oligonucleotides [103]. The reason for the stabilization afforded by N3'→P5' phosphoramidates is not clear, but most likely results form the altered configuration of the backbone, which favours triplex formation [100].

Triplex Binding Ligands

A further means for stabilizing intermolecular DNA triplexes is to use compounds which bind selectively to triplex and not duplex DNA. The first triplex-binding ligands were well known duplex intercalators such as ethidium [104], which preferentially bound to duplex DNA. More recently polycyclic compounds have been developed which selectively bind to triplex and not duplex DNA. The structure of several such agents are shown in Fig. (**7**). The first of these to be described was the benzo[*e*]pyridoindole (BePI) [105], which binds to triplex DNA by intercalation [106]. This agent, together with most other triplex ligands, selectively stabilizes regions of T•AT triplets rather than C^+•GC, presumably because the positive charge on the protonated cytosine prevents binding of the cationic ligand. The selectivity for triplex DNA is thought to arise from the large ring structure, which shows good overlap with the surrounding triplets and which is too large to stack efficiently into duplex DNA. On the basis of molecular modelling studies the side chain of BePI is thought to lie in the major groove between the duplex pyrimidine strand and the third strand (the Watson-Hoogsteen groove) [106]. Several derivatives of BePI have been evaluated for triplex binding activity by Hélène and coworkers who have shown similar good activity with benzo[*g*]pyridoindoles (BgPI) [107]. The side chains of BePI and BgPI have different effects on their activity; for BePI the chain decreases triplex formation whereas it is important for the action of BgPI. It has been suggested that with BgPI these lie in the major groove between the purine strand of the duplex and the third strand (the Crick-Hoogsteen groove) [107]. BgPI possesses a more linear structure than the crescent shaped of BePI, suggesting that the lower base stacking must be compensated by important electrostatic interactions. In contrast benzo[*f*]pyridoindole does not stabilise triplexes. Although most studies have examined the interaction of triplex-binding ligands with parallel triplexes BePI has also been shown to stabilize GT-containing triplexes designed to bind in the antiparallel (but not parallel) orientation [108]. Further studies on related fused aromatic systems have also shown that benzo[*f*]pyridoquinoxaline is also a selective triplex-binding ligand [109]. This series of ligands has been extended to include five membered ring systems such as benzo[*f*]quinoquinoxaline [110] and dibenzophenanthrolines [111]. The latter appears to be the most effective triple-binding ligand in this series described to date. The mono-substituted dibenzophenanthroline derivatives are more active than their disubstituted analogues.

A further compound which has been shown selectively to stabilise triplexes is the berberine alkaloid coralyne [112, 113], Fig. (**7**) which contains four fused aromatic rings. Early studies suggested that this ligand was effective at both C^+•GC and T•AT, though this has since been disputed [114]. Our recent studies have shown that a series of 2,6 disubstituted amidoanthraquinones successfully stabilised triplexes while their 1,4 counterparts only bound to duplex DNA [115]. The different properties of the two series of

compounds are thought to arise from differences in the stacking of the base triplets with the chromophore, combined with DNA groove accessibility. Confirmation of this binding selectivity and a thermodynamic basis for the different triplex/duplex preferences of the 2,6 and 1,4 isomers was provided by calorimetric and spectrophotometric techniques [116]. This study revealed that the 1,4 compounds destabilize DNA triplexes, leading to dissociation of the third strand, whereas their 2,6 isomeric analogues bind to and stabilize triple-stranded structures. A related study has also shown that selective stabilization of triplex DNA can be achieved with a series of disubstituted anthraquinone sulfonamides and suggested that 2,7-functionalised compounds are more potent than their 2,6 counterparts [117]. We have also compared the activity of four di-substituted and two monosubstituted amidoanthraquinone compounds, and have examined how the position of the cationic substituents affects triplex affinity [118]. Triplex affinity for the different substituent patterns decreases in order 2,7 > 1,8 = 1,5 > 2,6, with the equivalent monosubstituted compounds being at least an order of magnitude less efficient.

Naphthylquinoline derivatives, Fig (**7**) also selectively bind to triplex DNA by intercalation [119-123]. These compounds possess a large aromatic area to stack with the three bases in the triplex, yet, since the aromatic portions are not fused, they possess torsional flexibility and can accommodate the propeller twist of the triplets in which the three bases may not be coplanar. These compounds are also selective for T•AT over C^+•GC and are more effective at parallel than antiparallel triplexes [121]. A recent study has also suggested that a naphthylquinoline dimer may bind by bis-intercalation [124].

Triplex-binding ligands have also been used for increasing the strength of binding to sites which contain pyrimidine interruptions. In general these ligands do not affect the stringency of triplex formation, and the relative binding strengths of different oligonucleotide substitutions are not affected [122]. However by increasing the strength of binding, by up to 1000-fold, complexes can be formed at sequences for which there are no clear rules. The naphthylquinoline derivatives have been shown to promote the formation of triplexes at sites containing up to three consecutive base pair inversion using T•CG and G•TA triplets [125]. Studies with BePI have shown that the least destabilizing triplets are the same in both the presence and absence of the ligand and that the third strand base is less important in the presence of the ligand [126]. However addition of the ligand does provide some discrimination between different inverted base pairs. In particular 3-nitropyrrole discriminates CG from GC, TA and AT pairs in the presence, but not the absence of BePI [126]. Duplex regions of $(AT)_n$ have also been targeted with GT-containing oligonucleotides in the presence of the naphthylquinoline triplex-binding ligand, generating complexes containing alternating G•TA and T•AT triplets [127]. Although blocks of alternating T•AT and G•TA triplets alone are not stable, even in the presence a triplex-binding ligand, these complexes can be stabilized by attaching this region to a block of consecutive T•AT triplets, to which the ligand is thought to bind. In this way the duplex $A_{11}(AT)_6.(AT)_6T_{11}$ can be targeted with the third strand $T_{11}(TG)_6$, though this interaction still requires the presence of Mn^{2+} or a triplex binding ligand. These complexes can be extended to longer $(AT)_n$ tracts (up to n = 11) [128] and can be stabilised with shorter T•AT tracts. It should also be noted that these complexes can be further stabilized by including a few C^+•GC triplets in the anchoring tail and form in the presence of Mg^{2+}, without addition of a stabilizing ligand [128].

Tethered DNA Binding Agents

One of the first methods to be attempted for increasing triplex stability was to attach a DNA binding ligand to one end of the oligonucleotide. In this way sequence selectivity is achieved by the triplex forming oligonucleotide while the ligand acts as a non-selective anchor increasing the affinity. Most of these studies have used duplex intercalators as DNA binding ligands, though some recent examples have employed triplex binding ligands.

Intercalators

Several groups have shown that covalent attachment of an acridine moiety (usually [2-methoxy,6-chloro,9-amino]acridine) to the end of an oligonucleotide significantly increases triplex stability [129-133]. The acridine is proposed to intercalate at the triplex-duplex junction. Attachment to the 5'-end has a greater effect than at the 3'-end [130, 134], possibly reflecting a structural difference between the 5'-and 3'-triplex-duplex junctions, since the junction at the 5'-end of the third strand is thought to present a strong intercalation site [135, 136]. Most of these studies have used parallel, pyrimidine-containing oligonucleotides, though they have also been used to stabilise antiparallel GA and GT-containing triplexes [92, 137-140] as well as alternate strand triplexes containing both parallel and antiparallel motifs [141]. Attachment generally increases triplex stability by at least 100-fold, though the interaction with related non-cognate sequences is also increased [133]. However attachment of the acridine does not seem substantially to compromise the sequence specificity of binding [139]. At secondary sites where more than one binding

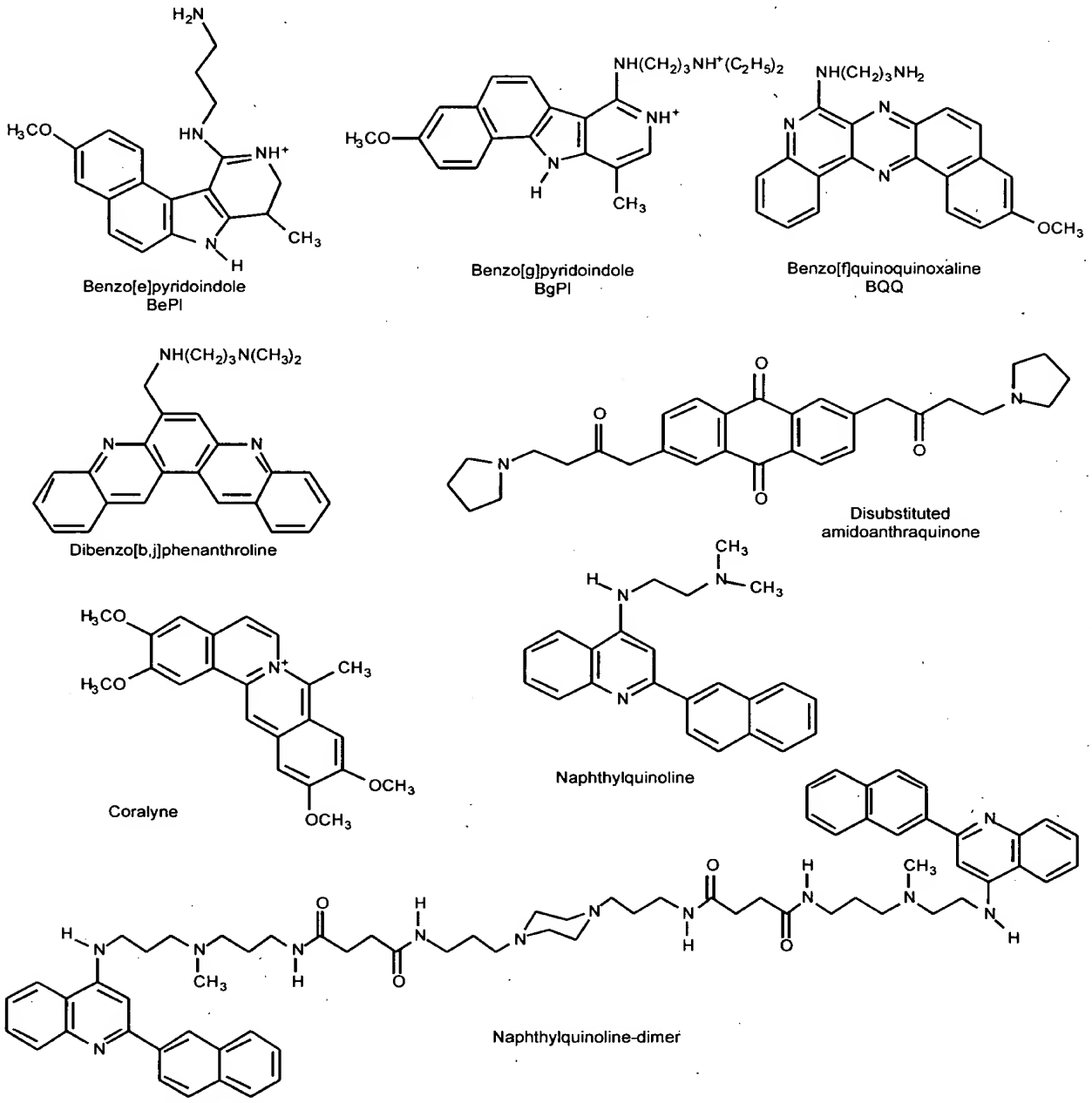

Fig. (7). DNA triplex-binding ligands.

mode is possible the oligonucleotide appears to bind so as to position the mismatches furthest from the intercalating moiety [133]. For GA-containing oligonucleotides covalent attachment of acridine partially overcomes the inhibitory effect of potassium on triplex formation [138], though GA containing oligonucleotides are still superior to those containing GT-residues [139].

Other intercalating agents which have been successfully linked to third strand oligonucleotides include orthophenanthroline [135], anthraquinone derivatives [142] and ellipticine [143].

Cross-linking Agents

The stability of DNA triplexes can also be increased by tethering a chemically reactive group to one or other end of the third strand oligonucleotide. The earliest experiments of this type used azidoproflavin [4] or azidophenacyl [144] to form monofunctional adducts. Bifunctional DNA cross-links can be formed upon long

wavelength irradiation of psoralen, conjugated to an oligonucleotide via either its 4' or 5 position, and can be used as a means of covalently attaching a triplex-forming oligonucleotide to its target site. This not only achieves selective gene inactivation, but provides a means for delivering a tethered mutagen to a target site, for the introduction of site-specific DNA damage. The most reactive sequence for photocrosslinking by psoralen is TpA, linking the thymines across the two strands [145]. Psoralen-linked oligonucleotides are therefore effective at triplex sites which are bordered by a TpA step. The first experiments of this type attached psoralen to the 5'-end of pyrimidine-containing third strand oligonucleotides [146, 147]. In this way successful complex formation was demonstrated at a 16 base oligopurine tract in the HIV proviral sequence at pH 6.0, even though this target sequence contained a run of six contiguous GC base pairs. However at elevated oligonucleotide concentrations secondary binding was observed at a site containing only eight matching base pairs, suggesting that the covalent reaction might induce the formation of some non-productive complexes. Binding was further increased by replacing the third strand cytosines with guanines forming parallel G•GC triplets. Similarly a 20 base pair sequence within the aromatase gene has been targeted with a psoralen-linked oligonucleotide, even though this polypurine tract is interrupted by three CG base pairs, for which T•CG triplets were employed [148, 149].

Psoralen-linked GA-containing oligonucleotides have also been used to introduce a mutation into a selected triplex target sequence. Most of the mutations detected are TA → AT transversions and are targeted at the psoralen intercalation site [151-152]. Mutations are generated by transcription-coupled repair [153] and involve the error-prone repair pathway, either alone or in combination with nucleotide excision repair [154]. This mechanism may depend on the sequence context since other studies have shown that a 19-mer GT-containing oligonucleotide with a 5'-psoralen is not repaired [155]. Double psoralen adducts, located at both ends of a triplex-forming oligonucleotide cannot be repaired efficiently in human cells [156]. The efficiency of triplex formation and gene inactivation can be further increased by tethering an acridine to the other end of the oligonucleotide [157].

Other DNA-binding agents which have been successfully tethered to triplex-forming oligonucleotides include the AT-selective minor groove binding ligand Hoechst 33258 [158, 159], the topoisomerase I inhibitor camptoethecin [160], duocarmycin [161], the DNA cleavage agent bleomycin [162] and a platinum-containing DNA cross-linking agent [163].

Tethered Triplex-binding Ligands

Benzopyridoindole and benzopyridoquinoline triplex-binding ligands have been attached to either the 5'-end or to an internucleotide position of triplex-forming oligonucleotides [164, 165]. For internal attachment the ligands were incorporated as an additional nucleotide, rather than replacing an existing one. All the derivatives stabilised triple helical structures more than either attachment of an acridine moiety or addition of the free ligand at an equivalent concentration. Comparison of their effect on two different parallel triplexes showed that the attached ligand had a greater stabilising effect on the sequence containing a longer run of contiguous thymines. Benzo[*h*]quinoquinoxaline was the most effective compound when attached to the 5'-end of the third strand, while BePI was the best ligand for the internal site. In addition these modifications did not alter the stringency of triplex formation, and introduction of a single mismatch caused a large decrease in binding affinity. We have also prepared oligonucleotides with a naphthylquinoline-derivative tethered to the 5'-end and have shown that these successfully stabilise triplexes to a greater extent than an equivalent concentration of free ligand [166]. It should also be noted, however, that with the tethered complex there is only one ligand stabilising each triplex, whereas with the free ligand several molecules may be able to bind simultaneously at different positions along the triplex.

Inclusion of a duplex binding agent within the third strand oligonucleotide has also be used to increase binding to sites containing pyrimidine interruptions, by placing an internal acridine group adjacent to the base facing the inverted pyrimidine.purine base pair [167, 168]. In this way, the loss of triplex stability at the inversion is partly overcome by the additional binding free energy of the intercalator, in a similar fashion to that achieved by attaching an intercalator to the 5'-end of the third strand. In the absence of a base inversion inclusion of an internal acridine has little or no effect on triplex stability, possibly because it is a duplex- rather than a triplex-specific intercalator. For targets containing CG or TA inversions the acridine moiety increases the stability of triplexes with either natural or synthetic bases opposing the pyrimidine base. Recognition of the TA base pair is strongest using either acridine or propanediol with an acridine on its 3'-side. Recognition of CG is greatest with either cytosine with acridine on its 3'-side or guanine with acridine on its 5'-side [167]. Similarly acridine-conjugated oligonucleotides have been used to stabilize the formation of triplexes at oligopurine tracts containing pyrimidine interruptions [140]. In these studies various natural or synthetic base analogues were placed opposite the pyrimidine residue, while the acridine was tethered to the 5'-end. These base 'mismatches' had a

greater destabilizing effect when place close to the intercalator end of the oligonucleotide.

Antiparallel Triplexes

Since pyrimidine-containing oligonucleotides, forming parallel DNA triplexes, require conditions of low pH, necessary for protonation of the third strand cytosine, it might be expected that purine-containing oligonucleotides designed to for antiparallel triplexes would be superior. Nonetheless these have not been so widely used for several reasons. Firstly the stability of antiparallel triplexes seems to vary widely from one study to another. Secondly these structures are dominated by G.GC, which usually constitutes over 60% of the triplets present, and G-rich oligonucleotides are known to adopt unusual structures which compete for triplex formation. Thirdly there are fewer studies on the recognition of pyrimidine interruptions and there is no specific means of recognising TA (see below).

Stability

In some studies antiparallel triplexes are shown to have very high affinities; short oligonucleotides exhibit nanomolar binding constants [169, 170], while in other studies longer oligonucleotides, under similar conditions, fail to show binding at micromolar concentrations [137, 170-173]. The simplest explanation for this differences is that the formation of antiparallel triplexes is very sequence dependent. This may be due to the different positions of the third strand backbone in G•GC, A•AT and T•AT triplets; unlike parallel T•AT and C^+•GC triplets which are isohelical. The most stable complexes are likely to be those with the lowest number of ApG and GpA steps in the homopurine target site. This sequence dependence is further compounded by the observation that the affinity of antiparallel triplexes is dominated by the G•GC triplet, which imparts a much greater stability that either A•AT or T•AT [137]. However, G-rich oligonucleotides form strong inter- and intramolecular structures, possibly involving G-quartet formation, which reduce the effective concentration of the oligonucleotide by competing with triplex formation. The best antiparallel triplexes may therefore be those in which the third strand has a high G-content, but in which the guanines are arranged in such a way as to prevent the formation of unusual structures.

There have been few studies directly comparing the stability of triplexes formed with CT- GT- and GA-containing oligonucleotides. It is generally agreed that GT triplexes are less stable than their GA counterparts [171, 174-176], possibly because the antiparallel G•GC triplet is more closely isomorphous to A•AT than reverse Hoogsteen T•AT [11]. The kinetics of triplex formation are also faster for antiparallel than parallel complexes [174-177].

Length

Although triplex stability generally increases with the length of the third strand, this is not necessarily the case for antiparallel triplexes. Cheng & Van Dyke [178] showed that, for the GT-motif short third strands (12-mers) could produce more stable complexes than longer oligonucleotides and that, in general, extensions and mutations at the 3'-end had greater effect than those at the 5'-end. Based on this observation they suggested that antiparallel triplex formation proceeded in the 3'→5' direction. Similarly in a REPSA selection assay for oligonucleotides which generate stable triplexes at a 19-mer target site, perfect matches were observed for the 13 bases at the 3'-end, while mismatches were selected at the 5'-end, and shorter oligonucleotides generated complexes with much reduced stability [179]. The greater effect of the 3'-end of the third strand has also been observed in footprinting experiments with oligonucleotides containing $(GGA)_n$ repeats, for which slipped structures were observed [171]. More recently this effect has been confirmed by Arimondo *et al.* [180] who compared the strength of triplex formation by $(AGG)_4$ and $(GGA)_4$. Although both complexes contained 4 x A•AT and 8 x G•GC triplets the oligonucleotide with a 3'-G [*i.e.* $(AGG)_4$] bound about 6-times better than the one with a 3'-A [*i.e.* $(GGA)_4$]. The addition of A•AT and G•GC triplets to the 5'-end of $(AGG)_4$ caused an increase in triplex stability as expected. In contrast, although the addition of a single G to the 3'-end of $(GGA)_4$ increased stability by about 10-fold, the addition of further As caused a decrease in stability. Furthermore overhanging mismatched bases had a much greater effect on stability when added to the 3'- than the 5'-end. Taken together these results suggest that antiparallel triplex formation proceeds from the 3'-end of the third strand and that nucleation is more efficient when the first base is a guanine.

Self Association

G-rich oligonucleotides are known to be able to form stable G-quartet structures, which compete for triplex formation in the presence of physiological concentration of monovalent cations, particularly K^+ [181, 182]. In addition GA- (but not GT) containing oligonucleotides can form homoduplexes, stabilised by internal GA repeats [183, 184]. The situation is further complicated by the observation that different inter- or intra-molecular structures can be formed with different sequences of (G,A)-containing oligonucleotides [185].

Self association of these oligonucleotides can effectively compete with triple helix formation and prevent complete triplex formation since, at high concentrations, more of the oligonucleotide is trapped in self-associated structures. The self-structures adopted by GA-containing oligonucleotides explain the opposite temperature dependence of triplexes formed with GA- and GT-containing oligonucleotides. The binding of GT-oligonucleotides decreases on raising the temperature from 4 to 37°C, in contrast to GA oligonucleotides for which the binding was increased, as the self-associated structure become less stable [183, 184].

Development of conditions which decrease the ability of G-containing oligonucleotides to form unusual structures will greatly improve our understanding of the formation of antiparallel triplexes. Phosphorothioate GA-containing oligonucleotides form less stable self-associated structures [92], but these form less stable triplexes than their phosphdiester counterparts. Divalent metal ions also alter the aggregation of G-rich sequences. Divalent metal ions, usually magnesium, are essential for forming antiparallel triplexes, though their effect is inhibited by physiological concentrations of K^+. However, the competition between triplex and self-associated structures is altered with other transition metal ions, and Co^{2+}, Mn^{2+}, and Ni^{2+} can promote triplex formation [186]. Other studies have attempted to overcome this problem by using base analogues which retain the ability to form Reverse-Hoogsteen hydrogen bonds, but which can no longer self associate. Replacement of guanine N7 with carbon, forming 7-deazaguanine, eliminates the formation of G-quartets, but decreases the ability to form triplexes [187]. Similarly several studies have replaced the guanine O6 with sulphur using 6-thioguanine [182, 188, 189]. This also reduces the binding affinity of the third strand, though it permits triplex formation in the presence of 200 mM K^+. An alterative strategy using 7-deazaxanthine (c^7X) in place of A or T in antiparallel oligonucleotides also achieved high affinity triplex formation under physiological potassium concentrations [187, 190].

Another approach uses the formation of a short duplex at either the 3'- or 5'-end of the third strand to prevent self association of GA oligonucleotides [191]. In these studies a 20-mer third strand was annealed with 10-17-mer oligonucleotides which were complementary to one or other end. These 'zipper' oligonucleotides still form a triplex over the entire length of the target site, suggesting that the short complementary strand has become 'unzipped'. This duplex region can include a large portion of the third strand and the minimal exposed single-stranded region can be as short as three nucleotides at the 3'-end or six nucleotide at the 5'-end. Examples of such 'zipper' oligonucleotides are shown in Fig. (**8**). The shorter length required at the 3'-end is consistent with the suggestion that triplex nucleation occurs in the 3'→5' direction.

Future Prospects

The work described above indicates that an enormous number of DNA analogues have been synthesized as potential triplex agents in the last 10 years. In addition there is an even greater literature on other analogues which have been used for developed as antisense agents. Is it possible to use these studies to define some of the features which are necessary for triplex formation which might guide the design of further derivatives with improved activity? On the basis of the results described above I will suggest possible developments for generating stable parallel, and antiparallel triplexes as well as some more general features which may improve triplex stability. These various modifications are summarised in Fig. (**9**).

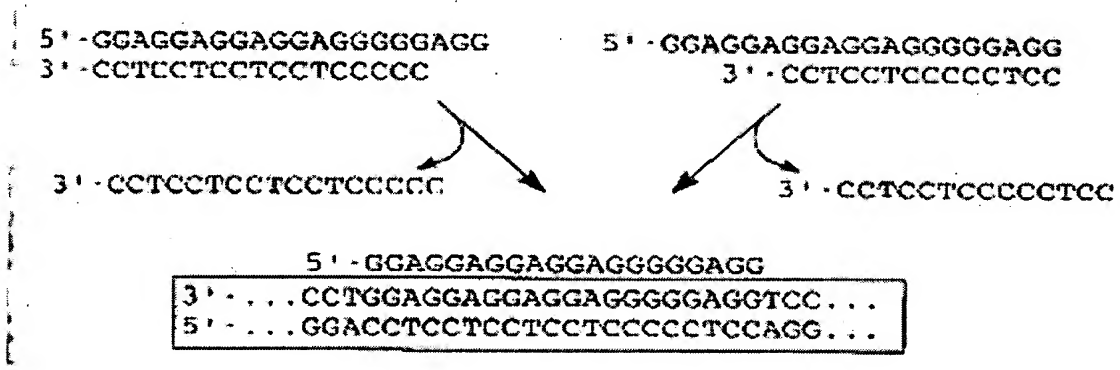

Fig. (8). Examples of 'zipper' oligonucleotides used to prevent self-association of guanine-containing third strands. The sequences were taken from [191].

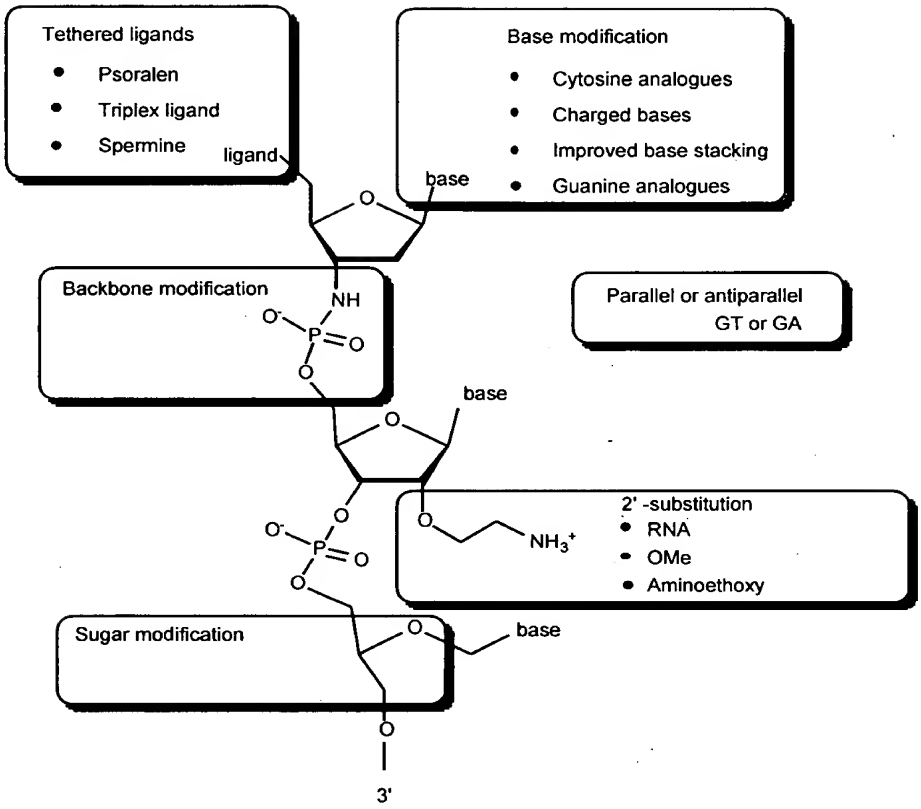

Fig. (9). Summary of potential sites of oligonucleotide modification for generating triplexes with enhanced stability.

Parallel Triplexes

One major problem for generating stable parallel triplexes under physiological conditions is the pH dependency of the C$^+$•GC triplet. Although several analogues have been shown to alleviate this problem none of these has found widespread use, possibly because of their limited availability, but also because of the large number of choices. In addition although many analogues remove the pH dependency they generate less stable triplexes than those formed with C$^+$•GC at low pH. Several features seem important in this regard. Firstly, since it is known that C$^+$•GC is more stable than T•AT [18-21], novel cytosine analogues with a hydrogen bond donor at a position equivalent to N3 should retain the positive charge either on the ring system, as in 2-aminopyridine [38-41], or added to a side group which may be attached to either N4 or C5 [59-62]. The importance of the positive charge suggests that the stability of T•AT might be improved by generating a charged analogue of T. However, since adjacent C$^+$•GC triplets are known to be destabilising, due to repulsion between the charged groups, it may be necessary to prepare both charged and uncharged analogues of T and C, and determine the optimum arrangement of the charged derivatives. Secondly it is necessary that the X•GC triplet should be isostructural with T•AT so as to minimise any distortions in the third strand backbone between adjacent base analogues. This may not be possible with purine analogues for recognition of GC when used alongside T•AT triplets, and similar derivatives, such as 2-aminopurine [45] or 8-oxoguanine may need to be developed for recognition of AT in these contexts. Thirdly triplex stability may be enhanced by increasing the base stacking in the third strand. However, simple addition of aromatic rings to the pyrimidine nucleus does not seem to be of benefit [76-79], possibly because the improved stacking of the single stranded oligonucleotide hinders triplex formation. Other stacking interactions, such as those involving appended propynyl or propargylamino groups [72-75], may be a better option. Lastly it is possible that different sugars should be used for third strand C or T, since it appears that rigid linkers improve the stability of T•AT, while flexible linkers improve the stability of C$^+$•GC [35, 36].

Antiparallel Triplexes

There have been fewer studies developing base analogues for use in antiparallel triplets, though there

are several outstanding problems which need to be addressed. Firstly there is the question of whether to use GA- or GT- containing oligonucleotide. GA sequences are more prone to self association [184, 185], yet the A•AT triplet is more closely isostructural to G•GC than T•AT [11]. Since these structures are dominated by the stability of the G•GC triplet, further analogues for isostructural recognition of AT (xanthine) may be useful. Secondly novel analogues (such as 6-thioguanine and 7-deazaguanine) will be needed to overcome the tendency of G-rich oligonucleotides to aggregate [188-190]. These limitations require further work optimizing the choice of third strand oligonucleotide. Thirdly, by analogy with C^+•GC, it may be beneficial to introduce positively charged groups onto the purine ring.

Stability

It is generally agreed that the lower stability of triplexes, relative to duplexes, can in part be attributed to charge repulsion between the three negatively charged phosphodiester backbones. Although several studies have shown that the introduction of positively charged groups into the backbone [59-66], sugar [70, 71] or bases [67-69, 72] can produce stable triplexes, these have usually been used in isolation. It is worth remembering that each triplet carries three negatively charged phosphates; so that yet more stable triplexes may be produced by combining several of these modifications in a single oligonucleotide. A further benefit of these modified oligonucleotides is that they often confer increased biological stability and resistance against nucleases. Triplex binding ligands offer a further method for increasing triplex stability which may also be useful when combined with some of the other approaches, or tethered to the end of the third strand. A further limiting factor for both triplex motifs is that there is still no good method for recognizing pyrimidine interruptions [15, 16]. This is more acute for antiparallel complexes for which there is no method for recognizing a TA base pair. Any novel bases for recognizing TA or CG will also need to be structurally compatible the existing method for targeting GC and TA. Triplex (or duplex) ligands may also be used for stabilizing weaker complexes across targets containing pyrimidine interruptions.

Although there have been many significant advances in the use of triplex forming oligonucleotides there are still many unanswered questions. Resolving these problems will require further close collaborations between chemists and molecular biologists before the potential of this strategy for targeting DNA is fully realised.

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References

[1] Felsenfeld, G.; Rich, A. *Biochim. Biophys. Acta* **1957**, *26*, 457.

[2] Felsenfeld, G.; Davis, D.R.; Rich, A. *J. Am. Chem. Soc.* **1957**, *79*, 2023.

[3] Moser, H.E.; Dervan, P.B. *Science* **1987**, *238*, 645.

[4] Le Doan, T.; Perrouault, L.; Praseuth, D.; Habhoub, N.; Decout, J.L.;Thuong, N.T.; Lhomme, J.; Hélène, C. *Nucleic Acids. Res.* **1987**, *15*, 7749.

[5] Soyfer, V.N.; Potaman, V.N. *Triple Helical Nucleic Acids* Springer-Verlag: New York, Berlin, **1996**.

[6] Thuong, N.T.; Hélène, C. *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 666.

[7] Vasquez, K.M.; Wilson, K.H. *Trends in Biochem.* **1998**, *23*, 4.

[8] Chubb, J.M.; Hogan, M.E. *Trends in Biotechnol.* **1992**, *10*, 132.

[9] Chan, P.P ; Glazer, P.M. *J. Mol. Med.* **1997**, *75*, 267.

[10] Neidle, S. *Anti-Cancer Drug Des.* **1997**, *12*, 433.

[11] Radhakrishnan, I.; Patel, D.J. *Structure* **1994**, *2*, 17.

[12] Beal, P.A.; Dervan, P.B. *Science* **1991**, *251*, 1360.

[13] Chen, F.-M. *Biochemistry* **1991**, *30*, 4472.

[14] Radhakrishnan, I.; Patel, D.J.*Structure* **1993**, *1*, 135.

[15] Doronina, S.O.; Behr, J.P. *Chem. Soc. Rev.* **1997**, *26*, 63.

[16] Gowers, D.M.; Fox, K.R. *Nucleic Acids Res.* **1999**, *27*, 1569.

[17] Lee, J.S.; Wordsworth, M.L.; Latimer, L.J.P.; Morgan, A.R. *Nucleic. Acids. Res.* **1984**, *12*, 6603.

[18] Roberts, R.W.; Crothers, D.M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, 93, 4320.

[19] Asensio, J.L.; Lane, A.N.; Dhesi, J.; Bergvist, S.; Brown, T. *J. Mol. Biol.* **1998**, *275*, 811.

[20] Keppler, M.D.; Fox, K.R. *Nucleic Acids Res.* **1997**, *25*, 4464.

[21] Volker, J.; Klump, H.H. *Biochemistry* **1994**, *33*, 13502.

[22] Giovannangeli, C.; Rougée, M.; Garestier, T.; Thuong, N.T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 8631.

[23] Xodo, L.E.; Manzini, G.; Quadrifoglio, F.; van der Marel, G.; van Boom, J. *Nucleic Acids Res.* **1991**, *19*, 5625.

[24] Povsic, T.J.; Dervan, P.B. *J. Am. Chem. Soc.* **1989**, *111*, 3059.

[25] Koh, J.S.; Dervan, P.B. *J. Am. Chem. Soc.* **1992**, *114*, 1470.

[26] Singleton, S.F.; Dervan, P.B. *Biochemistry* **1992**, *31*, 10995.

[27] Ono, A.; Ts'o, P.O.P.; Kan, L. *J. Am. Chem. Soc.* **1991**, *113*, 4032.

[28] Ono, A.; Ts'o, P.O.P.; Kan, L. *J. Org. Chem.* **1992**, *57*, 3225.

[29] Egholm, M.; Christensen, L.; Dueholm, K.L.; Buchardt, O.; Coull, J.; Nielsen, P.E. *Nucleic Acids Res.* **1995**, *23*, 217.

[30] Kuhn, H.; Demidov, V.V.; Frank-Kamenetskii, M.D.; Nielsen, P.E. *Nucleic Acids Res.* **1998** *26*, 582.

[31] Berressem, R.; Engels, J.W. *Nucleic Acids Res.* **1995**, *23*, 3465.

[32] Xiang, G.; Soussou, W.; McLaughlin, L.W. *J. Am. Chem. Soc.* **1994**, *116*, 11155.

[33] Xiang, G.; Bogacki, R.; McLaughlin, L.W. *Nucleic Acids Res.* **1996**, *24*, 1963.

[34] Xiang, G.; McLaughlin, L.W. *Tetrahedron* **1998**, *54*, 375.

[35] Tarkoy, M.; Leumann, C. *J. Org. Chem.* **1993**, 32, 1432.

[36] Jones, R.L.; Swaminathan, S.; Milligan, J.F.; Waswani, S.; Froehler, B.C.; Matteucci, M.D. *J. Am. Chem. Soc.* **1993**, *115*, 9816.

[37] von Krosigk, U.; Benner, S.A. *J. Am. Chem Soc.* **1995**, *117*, 5361.

[38] Bates, P.J.; Laughton, C.A.; Jenkins, T.C.; Capaldi, D.C.; Roselt, P.D.; Reese, C.B.; Neidle, S. *Nucleic Acids Res.* **1996**, *24*, 4176.

[39] Cassidy, S.A.; Slickers, P.; Trent, J.O.; Capaldi, D.C.; Roselt, P.D.; Reese, C.B.; Neidle, S.; Fox, K.R. *Nucleic Acids Res.* **1997**, *25*, 4891.

[40] Hildbrand, S.; Leumann, C. *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1968.

[41] Hildbrand, S.; Blaser, A.; Parel, S.P.; Leumann, C.J. *J. Am. Chem. Soc.* **1997**, *119*, 5499.

[42] Home, D.A.; Dervan, P.B. *Nucleic Acids Res.* **1991**, *19*, 4963.

[43] Froehler, B.C.; Ricca, D.J. *J. Am. Chem. Soc.* **1992**, *114*, 8320.

[44] Sun, J.-S.; De Bizemont, T.; Duval-Valentin, G.; Monteny-Garestier, T.; Hélène, C. *C.R.Acad. Sci. Paris Serie III* **1991**, *313*, 585.

[45] Roig, V.; Kurfurst, R.; Thuong, N.T. *TetrahedronLett.* **1993**, *34*, 1601.

[46] Miller, P.S.; Cushman, C.D. *Biochemistry* **1992**, *31*, 2999.

[47] Jetter, M.C.; Hobbs, F.W. *Biochemistry* **1993**, *32*, 3249.

[48] Krawczyk, S.H.; Milligan, J.F.; Wadwani, S.; Moulds, C.; Froehler, B.C.; Matteucci, M.D. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3761.

[49] Brunar H.; Dervan, P.B. *Nucleic Acids Res.* **1996**, *24*, 1987.

[50] Hunziker, J.; Priestley, E.S.; Brunar, H.; Dervan, P.B. *J. Am.Chem. Soc.* **1995**, *117*, 2661.

[51] Koshlap, K.M.; Schultze, P.; Brunar, H.; Dervan, P.B.; Feigon, J. *Biochemistry* **1997**, *36*, 2659.

[52] Koh, J.S.; Dervan, P.B. *J. Am. Chem. Soc.* **1992**, *114*, 1470.

[53] Radhakrishnan, I.; Patel, D.J.; Priestley, E.S.; Nash, H.M.; Dervan, P.B. *Biochemistry* **1993**, *32*, 11228.

[54] Priestley, E.S.; Dervan, P.B. *J. Am. Chem. Soc.* **1995**, *117*, 4761.

[55] St. Clair, A.; Xiang, G.; McLaughlin, L.W. *Nucleosides Nucleotides* **1998**, *17*, 925.

[56] Marfut, J.; Parel, S.P.; Leumann, C.J. *Nucleic Acids Res.* **1997**, *25*, 1875.

[57] Hampel, K.J.; Crosson, P.; Lee, J.S. *Biochemistry* **1991**, *30*, 4455.

[58] Thomas, T.; Thomas, T.J. *Biochemistry* **1993**, *32*, 14068.

[59] Barawkar, D.A.; Kumar, V.A.; Garesh, K.N. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 1665.

[60] Barawkar, D.A.; Rajeev, K.G.; Kumar, V.A.; Ganesh, K.N. *Nucleic Acids Res.* **1996**, *24*, 1229.

[61] Rajeev, K.G.; Jadhav, V.R.; Ganesh, K.N. *Nucleic Acids Res.* **1997**, *25*, 4187.

[62] Ganesh, K.N.; Rajeev, K.G.; Pallan, P.S.; Rana, V.S.; Barawkar, D.A.; Kumar, V.A. *Nucleosides Nucleotides* **1997**, *16*, 1271.

[63] Nara, H.; Ono, A.; Matsuda, A. *Bioconjugate Chem.* **1995**, *6*, 54.

[64] Tung, C.H.; Breslauer, K.J.; Stein, S. *Nucleic Acids Res.* **1993**, *21*, 5489.

[65] Sund C.; Puri, N.; Chattopadhyaya, J. *Tetrahedron* **1996**, *52*, 12275.

[66] Sund C.; Puri, N.; Chattopadhyaya, J. *NucleosidesNucleotides* **1997**, *16*, 755.

[67] Chaturvedi, S.; Horn, T.; Letsinger, R.L. *Nucleic Acids Res.* **1996**, 24, 2318.

[68] Dagle, J.M.; Weeks, D.L. *Nucleic Acids Res.* **1996**, *24*, 2143.

[69] Bailey, C.P.; Dagle, J.M.; Weeks, D.L. *Nucleic Acids Res.* **1998**, *26*, 4860.

[70] Cuenoud, B.; Casset, F.; Husken, D.; Natt, F.; Wolf, R.M.; Altmann, K.H.; Martin, P.; Moser, H.E. *Angew. Chem. Int. Ed. Engl.* **1998**, *37*, 1288.

[71] Blommers, T.J.J.; Natt, F.; Jahnke, W.; Cuenoud, B. *Biochemistry* **1998**, *37*, 17714.

[72] Bijapur, J.; Keppler, M.D.; Bergqvist, S.; Brown, T.; Fox, K.R. *Nucleic Acids Res.* **1999**, *27*, 1802.

[73] Colocci, N.; Dervan P.B. *J. Am. Chem. Soc.* **1994**, *116*, 785.

[74] Froehler, B.C.; Wadwani, S.; Terhorst, T.J.; Gerrard, S.R. *Tetrahedron Lett.* **1992**, *33*, 5307.

[75] Phipps, A.K.; Tarkoy, M.; Shultze, P.; Feigon, J. *Biochemistry* **1998**, *37*, 5820.

[76] Staubli, A.B.; Dervan, P.B. *Nucleic Acids Res.* **1994**, *22*, 2637.

[77] Michel, J.; Toulme, J.-J.; Vercauteren, J.; Moreau, S. *Nucleic Acids Res.* **1996**, *24*, 1127.

[78] Michel, J.; Gueguen, G.; Vercauteren, J.; Moreau, S. *Tetrahedron* **1997**, *53*, 8457.

[79] Godde, F.; Toulme, J.-J.; Moreau, S. *Biochemistry* **1998**, *37*, 13765.

[80] Semerad, C.L.; Maher L.J. *Nucleic Acids Res.* **1994**, *22*, 5231.

[81] Escudé C.; Francois, J.-C.; Sun, J.-S.; Oitt, G.; Sprinz, M.; Garestier, T.; Hélène, C. *Nucleic Acids Res.* **1993**, *21*, 5547.

[82] Han, H.; Dervan, P.B. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3806.

[83] Han, H.; Dervan, P.B. *Nucleic Acids Res.* **1994**, *22*, 2837.

[84] Roberts, R.W.;Crothers, D.M. *Science* **1992**, *258*, 1473.

[85] Noronha, A.; Damha, M.J. *Nucleic Acids Res.* **1998**, *26*, 2665.

[86] Wang, S.; Kool, E.T. *Nucleic Acids Res.* **1995**, *23*, 1157.

[87] Shimizu, M.; Konishi, A.; Shimada, Y.; Inoue, H.; Ohtsuka, E. *FEBS Lett.* **1992**, *302*, 155.

[88] Escudé,C.; Sun, J.-S.; Rougée, M.;Garestier, T.; Hélène, C. *C.R. Acad. Sci. Paris Série III* **1992**, *315*, 521.

[89] Kandimalla, E.R.; Venkataraman, G.; Sasisekharan, V.; Agrawal, S. *J. Biomol. Struct. & Dynam.* **1997**, *14*, 715.

[90] Latimer, L.J.P.; Hampel, K.; Lee, J.S. *Nucleic Acids Res.* **1989**, *17*, 1549.

[91] Hacia, J.G.; Wold, B.J.; Dervan, P.B. *Biochemistry* **1994**, *33*, 5367.

[92] Lacoste, J.; Francois, J.-C.; Hélène, C. *Nucleic Acids Res.* **1997**, *25*, 1991.

[93] Alunni-Fabbroni, M.G.; Manfioletti, G.; Manzini, G.; Xodo, L.E. *Eur. J. Biochem.* **1996**, *226*, 831.

[94] Kibler-Herzog, L.; Kell, B.; Zon, G.; Shinozuka, K.; Mizan, S.; Wilson, W.D. *Nucleic Acids Res.* **1990**, *18*, 3534.

[95] Kibler-Herzog, L.; Zon, G.; Whittier, G.; Mizan, S.; Wilson, W.D. *Anti-Cancer Drug Des.* **1993**, *8*, 65.

[96] Callahan, D.E.; Trapane, T.L.; Miller, P.S.; Ts'o, P.O.P.; Kan, L.-S. *Biochemistry* **1991**, *30*, 1650.

[97] Reynolds, M.A.; Arnold, L.J.; Almazan, M.Y.; Beck, T.A.; Hogrefe, R.I.; Metzler, M.D.; Stoughton, S.R.; Tseng, B.Y.; Trapane, T.L.; Ts'o, P.O.P.; Woolf, T.M. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 12433.

[98] Trapane, T.L.; Hogrefe, R.I.; Reynold, M.A.; Kan, L.S.; Ts'o, P.O.P. *Biochemistry* **1996**, *35*, 5495.

[99] Debart, F.; Meyer, A.; Vasseur, J.J.; Rayner, B. *Nucleic Acids Res.* **1998**, *26*, 4551.

[100] Escudé, C.; Giovannangeli, C.; Sun, J.-S.; Lloyd, D.H.; Chen, J.-K.; Gryaznov, S.M.; Garestier, T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4365.

[101] Zhou-Sun, B.W.; Sun, J.S.; Gryaznov, S.N.; Liquier, J.; Garestier, T.; Hélène, C.; Tailandier, E. *Nucleic Acids Res.* **1997**, *25*, 1782.

[102] Giovannangeli, C.; Perrouault, L.; Escudé, C.; Gryaznov, S.; Hélène, C. *J. Mol. Biol.* **1996**, *261*, 386.

[103] Gryaznov, S.M.; Winter, H. *Nucleic Acids Res.* **1998**, 26, 4160.

[104] Scaria, P.V.; Shafer, R.H. *J. Biol. Chem.* **1991**, *266*, 5417.

[105] Mergny, J.L.; Duval-Valentin, G.; Nguyen, C.H.; Perrouault, L.; Faucon, B.; Rougée, M.; Montenay-Garestier, T.; Nisagni, E.; Hélène, C. *Science* **1992**, *256*, 1681.

[106] Pilch, D.S.; Waring, M.J.; Sun, J.-S.; Rougee, M.; Nguyen, C.-H.; Bisagni, E.; Garestier, T.; Hélène, C. *J. Mol. Biol.* **1993**, *232*, 926.

[107] Escudé, C.; Nguyen, C.H.; Mergny, J.-L.; Sun, J.-S.; Bisagni, E.; Garestier, T.; Hélène, C. *J. Am. Chem. Soc.* **1995**, *117*, 10212.

[108] Escudé, C.; Sun, J.-S.; Nguyen, C.H.; Bisagni, E.; Garestier, T.; Hélène, C. *Biochemistry* **1996**, *35*, 5735.

[109] Escudé, C.; Nguyen, C.H.; Kukreti, S.; Janin, Y.; Sun, J.-S.; Bisagni, E.; Garestier, T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3591.

[110] Marchand, C.; Bailly, C.; Nguyen, C.H.; Bisagni, E.; Garestier, T.; Hélène, C.; Waring, M.J. *Biochemistry* **1996**, *35*, 5022.

[111] Baudoin, O.; Marchand, C.; Teulande, M.-P.; Vigneron, J.-P.; Sun, J.-S.; Garestier, T.; Hélène, C.; Lehn, J.-M. *Chem. Eur. J.* **1998**, *4*, 1504.

[112] Lee, J.S.; Latimer, L.J.P.; Hampel, K.J. *Biochemistry* **1993**, *32*, 5591.

[113] Latimer, L.J.P.; Payton, N.; Forsyth G.; Lee, J.S. *Biochem. Cell Biol.* **1995**, *73*, 11.

[114] Moraru-Allen, A.A.; Cassidy, S.; Alvarez, J.-L.A.; Fox, K.R.; Brown, T.; Lane, A.N. *Nucleic Acids Res.* **1997**, *25*, 1890.

[115] Fox, K.R.; Polucci, P.; Jenkins, T.C.; Neidle, S. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7887.

[116] Haq, I.; Ladbury, J.E.; Chowdhry, B.Z.; Jenkins, T.C. *J. Am. Chem. Soc.* **1996**, *118*, 10693.

[117] Kan, Y.Z.; Armitage, B.; Schuster, G.B. *Biochemistry* **1997**, *36*, 1461.

[118] Keppler, M.D.; Read, M.A.; Perry, P.J.; Jenkins, T.C.; Trent, J.O.; Reszka, A.P.; Neidle, S.; Fox, K.R. *Eur. J. Biochem.* **1999**, *263*, 817.

[119] Wilson, W.D.; Tanious, F.A.; Mizan, S.; Yao, S.; Kiselyov, A.S.; Zon, G.; Strekowski, L. *Biochemistry* **1993**, *32*, 10614.

[120] Cassidy, S.A.; Strekowski, L.; Wilson, W.D.; Fox, K.R. *Biochemistry* **1994**, *33*, 15338.

[121] Cassidy, S.A.; Strekowski, L; Fox, K.R. *Nucleic. Acids Res.* **1996**, *24*, 4133.

[122] Chandler, S.P.; Strekowski, L.; Wilson, W.D.; Fox, K.R. *Biochemistry* **1995**, *34*, 7234.

[123] Strekowski, L.; Gulevich, Y.; Baranowski, T.C.; Parker, A.N.; Kiselyov, A.S.; Lin, S.-Y.; Tanious, F.; Wilson, W.D. *J. Med. Chem.* **1996**, *39*, 3980.

[124] Keppler, M.D.; Zegrocka, O.; Strekowski, L.; Fox, K.R. *FEBS Lett.* **1999**, *447*, 223.

[125] Gowers, D.M.; Fox, K.R. *Nucleic Acids Res.* **1997**, *25*, 3787.

[126] Kukreti, S.; Sun, J.S.; Loakes, D.; Brown, D.M.; Nguyen, C.H.; Bisagni, E.; Garestier, T.; Hélène, C. *Nucleic Acids Res.* **1998**, *26*, 2179.

[127] Chandler, S.P.; Fox, K.R. *FEBS Letts.* **1995**, *360*, 21.

[128] Gowers, D.M.; Fox, K.R. *Nucleic Acids Res.* **1998**, *26*, 3626.

[129] Sun, J.C.; Giovannangeli, C.; Francois, J.C.; Kurfurst, K.; Monteny-Garestier, T.; Asseline, U.; Saison-Behmoaras, T.; Thuong, N.T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6023.

[130] Sun, J.-S.; Francois, J.-C.; Montenay-Garestier, T.; Saison-Behmoaras, T.; Roig, V.; Thuong, N.T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9198.

[131] Birg, F.; Praseuth, D.; Zerial, A.; Thuong, N.T.; Asseline, U.; Le Doan, T.; Hélène, C. *Nucleic Acids Res.* **1990**, *18*, 2901.

[132] Grigoriev, M.; Praseuth, D.; Robin, P.; Hemar, A.; Saiaon-Behmoaras, T.; Dautry-Varsat, A.; Thuong, N.T.; Hélène, C.; Harel-Bellan, A. *J. Biol. Chem.* **1992**, 267, 3389.

[133] Stonehouse T.J.; Fox, K.R. *Biochim. Biophys. Acta* **1994**, *1218*, 322.

[134] Montenay-Garestier, T.; Sun, J.S.; Chomilier, J.; Mergny, J.L.; Takasugi, M.; Asseline, U.; Thuoung, N.T.; Rougée, M.; Hélène, C. In: *Molecular Basis of Specificity in Nucleic Acid-Drug Interactions*, B.Pullman & J. Jortner (eds.) Kluwer Academic Publishers, Holland. **1990**, 275.

[135] Collier, D.A.; Mergny, J.-L.; Thuong, N.T. Hélène, C. *Nucleic Acids Res.* **1991**, *15*, 4219.

[136] Mouscadet, J.-F.; Ketterlé, C.; Goulaouic, H.; Carteau, S.; Subra, F.; Le Bret, M.; Auclair, C. *Biochemistry* **1994**, *33*, 4187.

[137] Fox, K.R. Nucleic Acids Res. **1994**, *22*, 2016.

[138] Gamper, H.B.; Kutyavin, I.V.; Rhinehart, R.L.; Lokhov, S.G.; Reed, M.W.; Meyer, R.B. *Biochemistry* **1997**, *36*, 14816.

[139] Klysik, J.; Kinset, B.M.; Hua, P.; Glass, G.A.; Orson, F.A. *Bioconjugate Chem.* **1997**, *8*, 318.

[140] Orson, F.M.; Klysik, J.; Bergstrom, D.E.; Ward, B.; Glass, G.A.; Hua, P.; Kinesy, B.M. *Nucleic Acids Res.* **1999**, *27*, 810.

[141] Washbrook, E.; Fox, K.R. *Biochem. J.* **1994**, *301*, 569.

[142] Gibson, V.; Anderson, R.J.; Brown, J.R.; Hartley, J.A.; Cassidy, S.A.; Fox, K.R.; Cairns, D. *Pharm. Sci.* **1996**, *2*, 49.

[143] Perrouault, L.; Asseline, U.; Rivalle, C.; Thuong, N.T; Bisagni, E.; Giovannangeli, C.; Le Doan, T.; Hélène, C. *Nature* **1990**, *344*, 358.

[144] Praseuth, D.; Perroualt, L.; Le Doan, T.; Chassignol, M.; Thouong, N.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1349.

[145] Sage, E.; Moustacchi, E. *Biochemistry* **1987**, *26*, 3307.

[146] Giovannangeli, C.; Thuong, N.T.; Hélène, C. *Nucleic Acids Res.* **1992**, *20*, 4275.

[147] Takasugi, M.; Guendouz, A.; Chassignol, M.; Decout, J.L.; Lhomme, J.; Thuong, N.T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5602.

[148] Macaulay, V.M.; Bates, P.J.; McLean, M.J.; Rowlands, M.G.; Jenkins, T.C.; Ashworth, A.; Neidle, S. *FEBS Lett.* **1995**, *372*, 222.

[149] Bates, P.J.; Macaulay, V.M.; McLean, M.J.; Jenkins, T.C.; Reska, A.P.; Laughton, C.A.; Neidle, S. *Nucleic Acids Res.* **1995**, *23*, 4283.

[150] Raha, M.; Wang, G.; Seidman, M.M.; Glazer, P.M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2941.

[151] Wang, G.; Levy, D.D.; Seidman, M.M.; Glazer, P.M. *Mol. Cell. Biol.* **1995**, *15*, 1759.

[152] Gasparro, F.P.; Havre, P.A.; Olack, G.A.; Gunther, E.J.; Glazer, P.M. Nucleic Acids Res. **1994**, *22*, 2845.

[153] Wang, G.; Seidman, M.M.; Glazer, P.M. *Science* **1996**, *271*, 802.

[154] Barre, F.-X.; Giovannangeli, C.; Hélène, C.; Harel-Bellan, A. *Nucleic Acids Res.* **1999**, *27*, 743.

[155] Musso, M.; Wang, J.C.; Van Dyke, M.W. *Nucleic Acids Res.* **1996**, *24*, 4924.

[156] Sandor, Z.; Bredberg, A. *FEBS Lett.* **1995**, *374*, 287.

[157] Duval-Valentin, G.M.; Takasugi, C.; Hélène, C.; Sage, E. *J. Mol. Biol.* **1998**, 278, 815.

[158] Robles, J.; Rajur, S.;McLaughlin, L.W. *J. Am. Chem. Soc.* **1996**, *118*, 5820.

[159] Rajur, S.B.; Robles, J.; Wiederholt, K.; Kuimelis, R.G.; McLaughlin, L.W. *J. Org. Chem.* **1997**, *62*, 523.

[160] Matteucci, M.; Lin, K.Y.; Huang, T.; Wagner, R.; Sternbach D.D; Mehrotra, M.; Besterman, J.M. *J. Am. Chem. Soc.* **1997**, *119*, 6939.

[161] Lin, C.H.; Patel, D.J. *J. Amer. Chem. Soc.* **1992**, *114*, 10658.

[162] Kane, S.A.; Hecht, S.M.; Sun, J.-S.; Garestier, T.;Hélène, C. *Biochemistry* **1995**, *34*, 16715.

[163] Colombier, C.; Lippert, B.; Leng, M. *Nucleic Acids Res.* **1996**, *24*, 4519.

[164] Silver, G.C.; Nguyen, C.H.; Boutorine, A.S.; Bisagni, E.; Garestier, T.; Hélène, C. *Bioconjugate Chem.* **1997**, *8*, 15.

[165] Silver, G.C.; Sun, J.-S.; Nguyen, C.H.; Boutorine, A.S.; Bisagni, E.; Hélène, C. *J. Am. Chem Soc.* **1998**, *119*, 263.

[166] Keppler, M.D.; McKeen, C.M.; Zegrocka, O.; Strekowski, L.; Brown, T.; Fox, K.R. *Biochem. Biophys. Acta* **1999**, in press.

[167] Zhou, B.; Puga; E.; Sun, J.; Garestier, T.; Hélène, C. *J. Am. Chem Soc.* **1995**, *117*, 10425.

[168] Kukreti, S.; Sun, J.S.; Garestier,T.; Hélène,C. *Nucleic Acids Res.* **1997**, *25*, 4264.

[169] Svinarchuk, F.; Bertrand, J.-R.; Malvy, C. *Nucleic Acids Res.* **1994**, *22*, 3742.

[170] Svinarchuk, F.; Paoletti, J.; Malvy, C. *J. Biol. Chem.* **1995**, *270*, 14068.

[171] Chandler, S.P.; Fox, K.R. *Biochemistry* **1996**, *35*, 15038.

[172] Brown, P.M.; Fox, K.R. *Biochem. J.* **1996**, *319*, 607.

[173] Brown, P.M.; Madden, C.A.; Fox, K.R. *Biochemistry* **1998**, *37*, 16139.

[174] Faucon, B.; Mergny, J.-L.; Hélène, C. *Nucleic Acids Res.* **1996**, *24*, 3181.

[175] Roy, C. *Nucleic Acids Res.* **1993**, *21*, 2845.

[176] Klysik, J.; Kinset, B.M.; Hua, P.; Glass, G.A.; Orson, F.A. *Bioconjugate Chem.* **1997**, *8*, 318.

[177] Paes, H.M.; Fox, K.R. *Nucleic Acids Res.* **1997**, *25*, 3269.

[178] Cheng, A.-J.; van Dyke, M.W. *Nucleic Acids Res.* **1994**, *22*, 4742.

[179] Hardenbol, P.; Van Dyke M.W. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2811.

[180] Arimondo, P.B.; Barcelo, F.; Sun, J.-S.; Maurizot, J.-C.; Garestier, T.; Hélène, C. *Biochemistry* **1998**, *37*, 16627.

[181] Cheng, A.-J.; Van Dyke, M.W. *Nucleic Acids Res.* **1993**, *21*, 5630.

[182] Olivas, W.M.; Maher, L.J. *Nucleic Acids Res.* **1995**, *23*, 1936.

[183] Noonberg, S.B.; Francoise, J.C.; Garestier, T.; Hélène, C. *Nucleic Acids Res.* **1995**, *23*, 1956.

[184] Noonberg, S.B.; Francois, J.-C.; Praseuth, D.; Guieyesse-Peugot, A.-L.; Lacoste, J.; Garestier, T.; Hélène, C. *Nucleic Acids Res.* **1995**, *23*, 4042.

[185] Lee, J.S. *Nucleic Acids Res.* **1990**, *18*, 6057.

[186] Blume, S.W.; Guarcello, V.; Zacharias, W.; Miller, D.M. *Nucleic Acids Res.* **1997**, *25*, 617.

[187] Milligan, J.F.; Krawczyk, S.H.; Wadwani, S.; Matteucci, M.D. *Nucleic Acids Res.* **1993**, *21*, 327.

[188] Gee, J.E.; Revankar, G.R.; Rao, T.S; Hogan, M.E. *Biochemistry* **1995**, *34*, 2042.

[189] Rao, T.S.; Durland, R.H.; Seth, D.M.; Myrick, M.A.; Bodpeudi, V.; Revankar, G.R. *Biochemistry* **1995**, *34*, 765.

[190] Faruqi, A.F.; Krawczyk, S.H.; Metteucci, M.D.; Glazer, P.M. *Nucleic Acids Res.* **1997**, *25*, 633.

[191] Svinarchuk, F.; Cherny, D.; Debin, A.; Delain, E.; Malvy, C. *Nucleic Acids Res.* **1996**, *24*, 385.

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Targeting DNA with Triplexes

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Abstract: The formation of intermolecular DNA triple helices offers the possibility of designing compounds with extensive sequence recognition properties which may be useful as antigene agents or tools in molecular biology. In these structures a third strand oligonucleotide binds in the DNA major groove, making specific contacts with substituents on the exposed faces of the base pairs. Although triplexes form with exquisite specificity their use suffers from several drawbacks. Two limitations of this approach, which are considered in this review are, firstly that conditions of low pH are necessary for formation of the C$^+$•GC triplet, and secondly that these structures are often less stable than their duplex counterparts. This review outlines the strategies that have been employed to overcome these drawbacks. The pH problem is addressed by considering the various DNA base analogues that have been used to recognise GC base pairs in a pH independent fashion, and discusses the benefits and limitations of each analogue. Triplex stability can be increased by using novel base analogues, backbone modifications and the use of triplex-specific binding ligands.

Background

Compounds which interact with duplex DNA in a sequence-specific fashion have the potential to inhibit the activity of individual genes, and may be useful for treating a variety of diseases including cancer or viral infections. In principle it should be simpler to design drugs targeted to DNA than against many other pharmacological receptors, since the structure of the target is well known in precise molecular detail. In addition there are only two copies of each DNA target site per diploid cell, and it may therefore be possible to use low doses of such agents, provided that they maintain high affinity and stringency. In order to be selective for a unique DNA sequence within the human genome of 3×10^9 base pairs, such agents need to recognise at least 16-17 consecutive bases. Sequence specific recognition of DNA is achieved by making specific contacts with the regular array of hydrogen bond donors and acceptors on the base pairs which are exposed in the major and minor grooves. Since these recognition elements are positioned in a helical arrangement, turning through about 360° every 10 base pairs, DNA sequence-reading agents need to follow this helical pattern. A versatile code for generating sequence-specific DNA-reading agents will therefore require monomer units which selectively bind to the individual bases (or base pairs), and which are attached to a regular backbone which is able to wind around the DNA helix, possessing the same axial rise as duplex DNA. A simple solution to these problems is to use oligonucleotides as sequence specific agents, since their repeating unit is the same as that of duplex DNA, and they can easily adopt a helical structure. It has been known for several years that the major groove of duplex DNA is large enough to accommodate a third oligonucleotide strand, forming an intermolecular triplex. By exploring the rules governing the formation of these complexes it is hoped to generate a versatile recognition code for designing agents to interact with any desired sequence.

The formation of DNA and RNA triple helices was first demonstrated in 1957 by mixing polyU and polyA in the ratio of 2:1 [1,2]. This and other triplexes, formed with synthetic polynucleotides, remained an obscure part of DNA chemistry until 1987 when it was realised that they offered a means for designing DNA sequence specific agents [3,4]. Since then a variety of biological activities have been proposed for these structures [5-10]. In these complexes the third strand lies in the major groove of the target DNA duplex, where it makes specific hydrogen bond contacts with substituents on the exposed faces of the duplex DNA base pairs. Two types of triplexes have been characterised, which differ in the orientation of the third strand. Those in which the third strand runs parallel to the duplex purine strand are characterised by T•AT and C$^+$•GC triplets, Fig. (1a),

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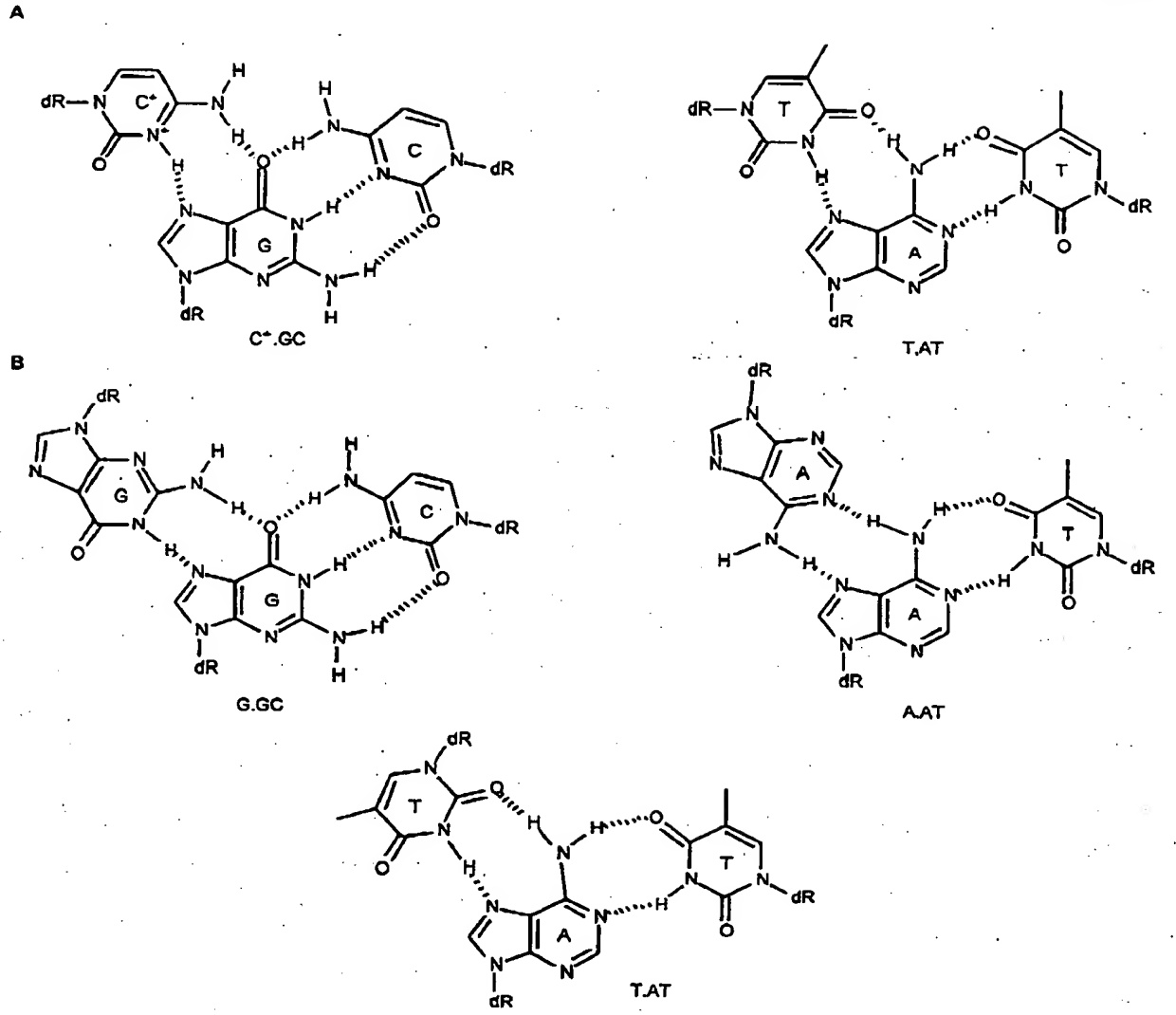

Fig. (1). A) Structures of the parallel triplets C$^+$•GC and T•AT. B) Structures of the antiparallel triplets G•GC, A•AT and T•AT.

and are stabilised by the formation of Hoogsteen base pairs [3,4,11]. Within this motif the C$^+$•GC triplet requires conditions of low pH (<6.0), necessary for protonation of the third strand cytosine. In contrast antiparallel triplexes are characterised by A•AT, T•AT and G•GC triplets, Fig. (**1b**) and are stabilised by reverse-Hoogsteen base pairs [12- 14]. [In the following, the notation X•ZY denotes a triplet in which the third strand base X interacts with a ZY base pair forming hydrogen bonds to base Z]. The details of the structures and applications of these standard triplexes have been considered in several recent reviews [4-10].

Although intermolecular triple helix formation offers the possibility of selectively targeting unique DNA sequences, it currently suffers from several limitations, two of which are considered in this review. Firstly, parallel triplex formation requires conditions of low pH necessary for formation of the C$^+$•GC triplet. Secondly, triplexes form with lower stability than their duplex counterparts. Triplex formation is also generally restricted to homopurine tracts, and this problem has been considered in recent reviews [15,16].

Cytosine Protonation

Parallel triplex formation generally requires conditions of low pH (< 6.0) necessary for protonation of N3 of the third strand cytosine in the C$^+$•GC triplet. Several cytosine analogues have therefore been designed with the aim of forming Hoogsteen hydrogen

bonds with guanine at physiological pHs. However, before describing these derivatives, it is first worth considering some of the essential features of the C^+•GC triplet, some of which are sometimes overlooked when designing new base analogues.

Firstly, although cytosine protonation requires conditions of low pH, and contiguous C^+•GC triplets are destabilising [17], several studies have shown that C^+•GC imparts a greater triplex stability than T•AT [18-21]. In contrast the unprotonated C•GC triplet, containing only one Hoogsteen hydrogen bond, is less stable than T•AT. The very large stabilisation from protonation is too large to be accounted for by the single additional hydrogen bond [19], and must therefore include contributions from either electrostatic interactions with the phosphate backbone or altered stacking with the neighbouring bases, possibly as a result of favourable interactions between the positive charge and the π-stack. Although the pK of free cytosine is about 4.5, this is elevated on triplex formation and may be as high as 9.0 for an isolated internal cytosine [19]. Terminal C^+•GC triplets are less stable, with lower pKs, presumably because the protonated base is exposed to solvent, and the interaction with only one nearest neighbour is less stabilising than when it can interact with two neighbours [19]. A consequence of the stronger binding of C^+•GC than T•AT is that N3-protonated cytosine analogues which retain the positive charge are likely to produce more stable triplexes than those in which the charge is missing. Additionally it may in future be worth considering the synthesis of charged thymine analogues so as to increase the stability of the T•AT triplet.

A second important factor is that, unlike the different triplet combinations in antiparallel triplexes, C^+•GC and T•AT triplets are isostructural [6, 22]. As a consequence there are no differences in backbone distortion between the various third strand base steps, i.e. TpC, CpT, TpT and CpC, and the base overlap for different steps is also constant. An inevitable consequence of using novel third strand cytosine analogues is that the X•GC triplet may no longer be compatible with T•AT. This may be especially relevant for analogues which are based on a purine rather than a pyrimidine ring. This disadvantage could of course be overcome by simultaneously introducing similarly modified thymine analogues generating X•AT triplets which are isostructural with X•GC.

The free cytosine nucleoside has a pK of about 4.5, though this is elevated at isolated cytosines within triplex forming oligonucleotides, depending on their number and location. Several base analogues have been synthesised in attempts to overcome this restriction, some of which are presented in Figs. (2) and (3). It can be seen that recognition of guanine requires a structure presenting two hydrogen bond

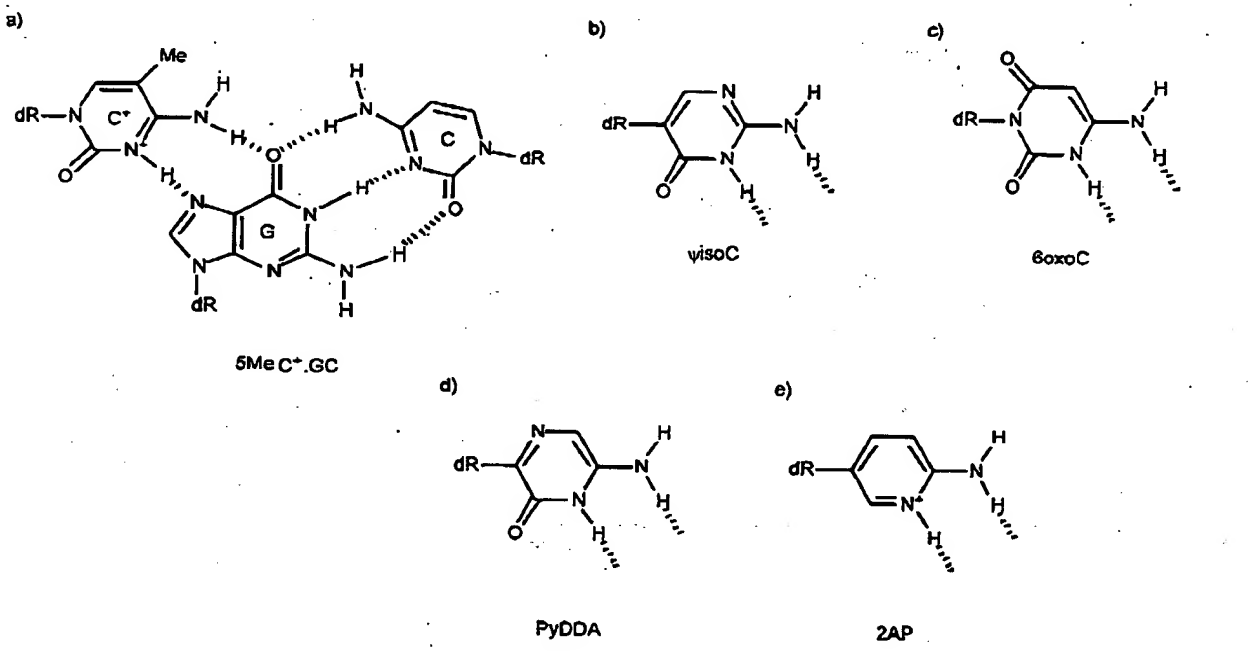

Fig. (2). Pyrimidine analogues for recognition of GC base pairs in a parallel triplex. a) the $^{5Me}C^+$•GC triplet, b) pseudo-isocytosine [27,28], c) 6-oxocytosine [31-34], d) pyrazine analogue [37], e) 2-aminopyridine [38-41]. In b) - e) the third strand base analogue is shown alone in the same orientation as in a), indicating the substituents that form hydrogen bonds to G.

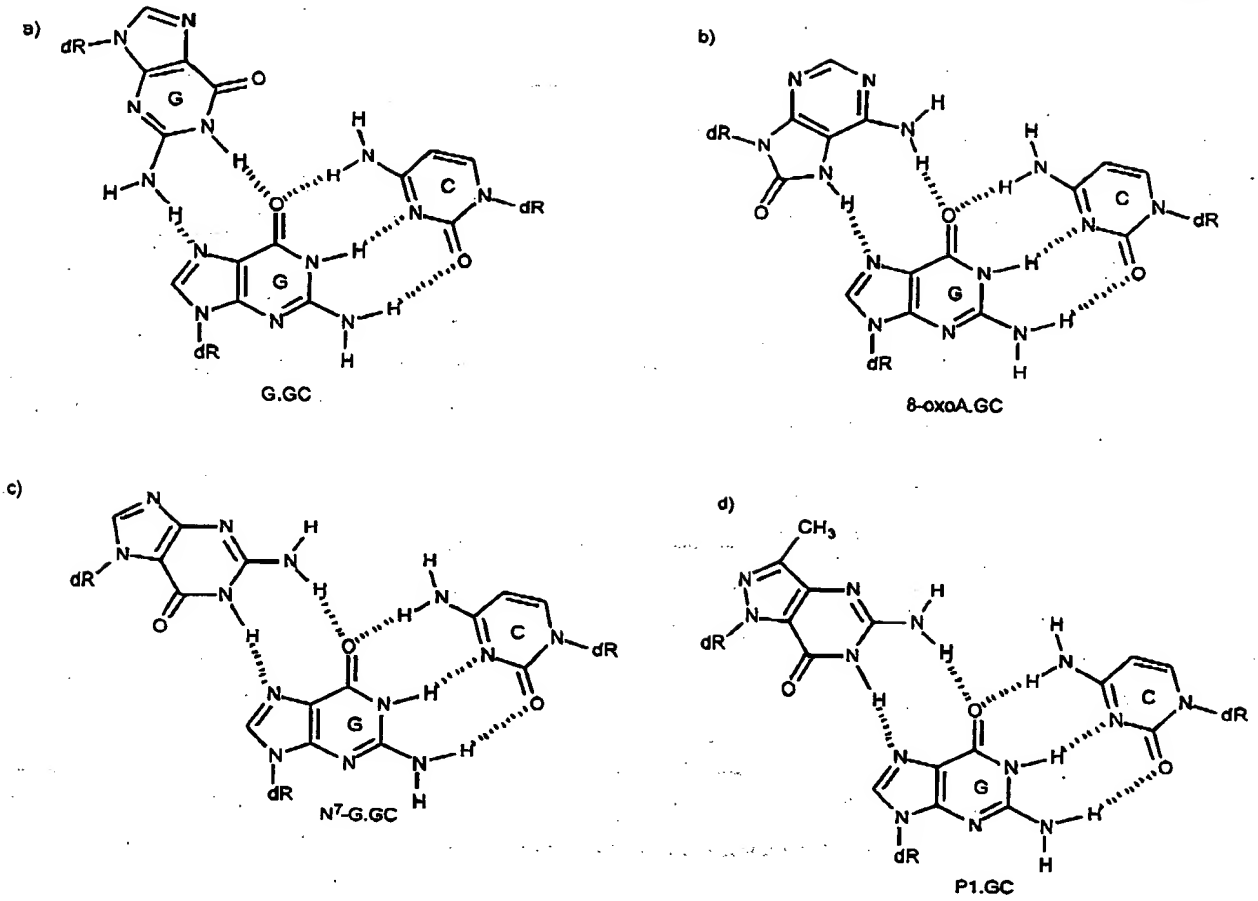

Fig. (3). Purine analogues for recognition of GC base pairs in a parallel triplex. a) the parallel G•GC triplet [20, 44], b) 8-oxoadenine•GC triplet [46-48], c) N^7-G•GC triplet, d) P1•GC triplet.

donors to interact with O6 and N7. These analogues can be divided into two classes which are based on either a pyrimidine or a purine ring structure.

Pyrimidine Analogues

The first cytosine analogue to be tested was 5-methylcytosine, Fig. (2a) a naturally occurring base, since this has a higher pK value than cytosine [17, 23, 24]. Triplexes containing this base are indeed more stable at slightly higher pHs, but are still not formed under physiological conditions [25]. The improvement in the pH profile of ^{5Me}C relative to C is better than would be expected from the small change in pK, and it has been suggested that the increased stability results from the extra spine of methyl groups within the DNA major groove. These methyl groups increase stability by either inducing the release of water molecules, thereby contributing a positive entropy change [23] or by improving the base stacking [26]. This improvement in pH profile is accompanied by an increase in affinity [24].

Pseudoisocytosine, Fig. (2b) [27,28] has been successfully employed, as its 2'-O- methyl derivative, for triplex recognition of guanine. In the tautomeric form shown in Fig. (2b) this base contains a hydrogen at position N3 and has the same potential for forming Hoogsteen hydrogen bonds with G as protonated cytosine. In the alternative tautomer N1 is protonated rather than N3 and it can form a stable base pair with guanine [28].This analogue has been shown to form pH-independent triplets with GC base pairs which are more stable than those formed with natural bases [28]. The formation of C^+•GC triplets is especially difficult in runs of contiguous GC base pairs, presumably because of unfavourable charge interactions between adjacent protonated cytosines, which further decreases their effective pK. Pseudoisocytosine, alone amongst the uncharged pyrimidine analogues (see below) appears to overcome this limitation and has been shown to form

a stable triplex at a site containing six contiguous guanines. Surprisingly this base has not found widespread use in triplex strategies, because of its limited availability and difficult synthesis, but it is more frequently employed as a cytosine analogue in PNA-containing structures [29, 30].

6-Oxocytosine, Fig. (**2c**) is a further pyrimidine analogue which presents the correct arrangement of hydrogen bonds for pH independent recognition of guanine [31-34]. Although this base forms a pH independent triplet with GC, the complexes generated are less stable than those observed with C or ^{5Me}C at low pH. This could reflect the importance of the positive charge noted above, affecting the energetics of base stacking, or might be due to changes in the hydrogen bonding characteristics since the natural bases form one hydrogen bond with a charged partner in contrast to the uncharged partner with the synthetic base. The less efficient base stacking observed with this analogue is consistent with the observation that this base does not form stable triplexes at blocks of contiguous guanines, even though it does not suffer from the charge repulsion seen with C$^+$ and ^{5Me}C$^+$ [33]. Surprisingly stable recognition of G-tracts is achieved by alternating this base analogue with ^{5Me}C [33]. More recently it has been shown that 6-oxocytosine forms a more stable triplet when deoxyribose is replaced with a flexible acyclic linker [34]. In contrast this flexible linker lowered the stability of T•AT triplets. It seems to be a common feature that T•AT triplets are stabilized by rigid linkers while C$^+$•GC requires a less rigid linker [35, 36]. This most likely reflects the different optimal sugar conformations and base stacking environments required for C$^+$•GC and T•AT. With 6- oxocytosine triplex stability decreased slightly as the number of acyclic linkers in the oligonucleotide was increased from 2 to 6, presumably because unfavourable entropy effects overcome the enhanced base stacking.

A further uncharged cytosine analogue for GC recognition is based on a pyrazine ring [37] and is shown in Fig. (**2d**). Although there is little information on this analogue it showed pH independent recognition of a single guanine embedded in an AT tract (as its 2'-O-methyl riboside).

Another promising cytosine analogue is 2-aminopyridine (2AP), Fig. (**2e**) which has a pK of 6.86, closer to physiological pH [38-41]. Psoralen-linked oligonucleotides containing this base have been successfully targeted against a portion of the aromatase gene [38]. This analogue forms stable triplexes at higher pHs than either C or ^{5Me}C [39]. This increased stability is also evident at low pHs suggesting that the 2AP•GC triplet is intrinsically more stable. This base analogue also forms stable complexes at blocks of contiguous GC base pairs. In footprinting experiments $(2AP)_6T_6$ formed a triplex with G_6A_6•T_6C_6 at pHs as high as 7.0, in contrast to $^{5Me}C_6T_6$ and C_6T_6, for which complexes are barely stable at pH 5.0 [39]. Surprisingly addition of a 5-methyl group to this base analogue does not affect its activity, and the 2'-O-methyl derivative is less active [41].

An alternative possibility for recognition of GC base pairs at physiological pHs is to retain the use of third strand cytosines, generating a triplet which is stabilized by only one hydrogen bond [42]. Since T•AT is isomorphous with C•GC, the third strand stacking interactions and phosphodiester backbone configurations may allow stable complex formation without the need for two hydrogen bonds in every C•GC triplet.

An alternative approach to base modification for altering the pK of cytosine is to modify the sugar. The pK of the carbocyclic derivative of ^{5Me}C is increased by 0.45 relative to ^{5Me}C and forms more stable triplets [43]. In contrast the carbocyclic analogue of T forms less stable T•AT triplets. This difference may reflect the different preferred sugar conformations adopted by C•GC and T•AT, since the carbocyclic sugar adopts a C1'-*exo* configuration.

Purine Analogues

A different strategy for GC recognition uses purine analogues to position the required hydrogen bond donors and several of these are shown in Fig. (**3**). It should however be noted that these analogues form triplets which are not isostructural with T•AT since the backbone must be in a different position. As a consequence they may need to be used in combination with equivalent analogues of T for recognition of AT pairs. Purine-rich oligonucleotides generally bind to duplex DNA in an antiparallel orientation generating G•GC, A•AT and (reverse Hoogsteen) T•AT triplets. However, it has been shown that GT-containing oligonucleotides can bind in a parallel configuration, Fig. (**3a**) though the strand orientation depends on the number of GpT and TpG steps [22, 44]. Similarly 2-amino-purine has been suggested for recognizing AT pairs in the parallel motif [45].

The first synthetic purine derivatives to be described were 8-oxoadenine [46, 47], Fig. (**3b**) and its N6-methyl analogue [48], which, in the *syn* conformation, are arranged so that the 6-amino and N7 protons replace the 4-amino and N3 protons of cytosine. These analogues have been shown to recognise guanine in a pH independent fashion forming triplets which are as stable as C$^+$•GC at low pH. 8-oxoA has been shown to form a stable triplex at a site

containing four contiguous guanines, which cannot be targeted with cytosine-containing oligonucleotides. Since this base has been tested in oligonucleotides containing T for recognition of AT pairs, it is clear that the third strand backbone must be distorted at each GA and AG step in the target. A more even backbone might be achieved by using this base in combination with 8-oxoguanine, which in the *syn* conformation, should be able to form Hoogsteen hydrogen bonds to A.

Other purine analogues, N7-G, Fig. (**3c**) [49-51], and P1, Fig. (**3d**) [52-54] bind in an *anti* configuration recognizing GC in a pH independent fashion. Although isolated P1.GC and N7-G.GC triplets show a similar stability to $^{5Me}C^{+}$•GC, recognition of alternating GA sequences by alternating P1 and T or N7-G and T is about three orders of magnitude lower than with oligonucleotides containing ^{5Me}C and T. In contrast, tracts of six contiguous guanines are bound by P1 or N7-G about four orders of magnitude better than ^{5Me}C. These differences are probably caused by the lack of structural isomorphism between N7-G•GC and T•AT or P1•GC and T•AT, whereas no such structural distortion is present within tracts of identical triplets. A similar effect is seen when N7-G is connected by a flexible acyclic linker [55]. The location of the phosphodiester backbone is seen to be important since on moving the glycosidic linkage in P1 from N7 to N8, generating base P2, no triplex formation was observed. These base analogues for GC recognition suggest that it may be possible to generate a new parallel stranded motif based on N7 purines.

A further analogue suggested for recognition of GC base pairs within the parallel motif is N7-inosine. It has been suggested that this base, which can be used for pH independent recognition of GC in both alternating GA and oligo G-tracts, binds by forming a single hydrogen bond between N1H of inosine and guanine N7. Interaction with other base pairs is prohibited by unfavourable repulsive Van der Waal's interaction [56].

Strength Of Binding

Although triplex-forming oligonucleotides bind with high specificity, their binding may not be strong and weaker than that of the underlying DNA duplex. In large part this is thought to be due to the charge repulsion resulting from bringing together the three polyanionic DNA strands. Several strategies, described below, have therefore been adopted for increasing triplex stability.

Positive Charges

One obvious approach for neutralising the charge repulsion between the three negatively charged phosphodiester backbones is to modify either the

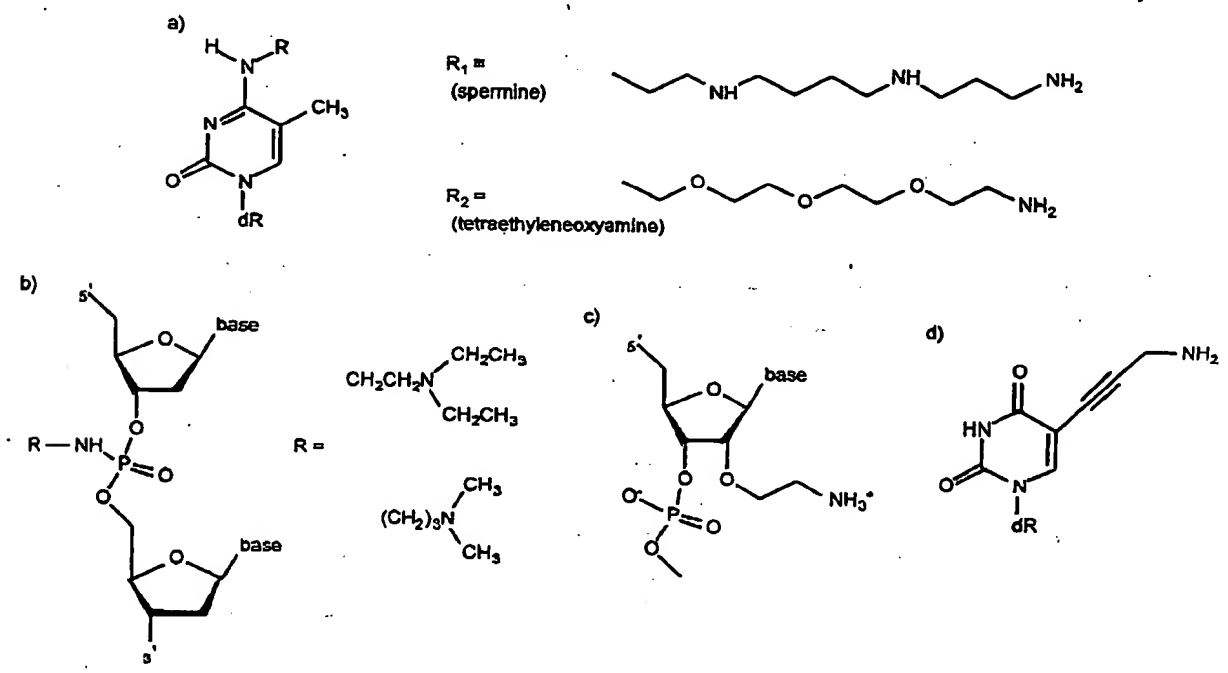

Fig. (4). Positively charged DNA analogues. a) N4-spermine derivatives of methylcytosine [59-62], b) cationic phosphoramidate backbone [67-69], c) 2'-aminoethoxy [70, 71], d) propargylamino-dU [72].

sugar-phosphate backbone or the bases so as to incorporate positive charges. Since triplexes are known to be stabilised by spermine [57, 58] several groups have covalently attached this group to N4 [59-62] of methylcytosine, Fig. (**4a**). Surprisingly these triplexes were stable at pH 7.4, even though N3 should be unprotonated at this pH, suggesting that the lack of the second hydrogen bond in the C^+•GC triplet can be compensated by favourable electrostatic interactions. Indeed these triplexes were less stable at lower pHs. This substitution was more effective when placed at either end of the triplex than at the centre and caused a slight decrease in Tm with increasing number of substitutions. In addition these triplexes were less dependent on the presence of divalent metal ions such as Mg^{2+}. Replacing the polycationic spermine with tetraethoxyleneoxyamine, Fig. (**4a**) produced a similar effect on triplex stability [61], presumably by virtue of favourable hydrophobic, rather than purely electrostatic interactions. Other studies with *syn*-norspermidine linked to the 5-position of U in the third strand also show significant triplex stabilization at physiological pH [63]. Spermine has also been attached to the 5'-terminus [64] and to the 2'-O of oligonucleotides [65, 66]. Both these modifications increase triplex melting temperatures, though 2'-O-linked spermine induced triplex formation when added at the 3'- or 5' end, but not at the centre of the third strand oligonucleotide.

Several studies have examined triplex formation by oligonucleotides containing neutral or positively charged backbones in attempts to alleviate the charge repulsion between the polyanionic strands. Examples of this include the cationic phosphoramidates shown in Fig. (**4b**). These have been shown to stabilise both parallel [67] and antiparallel triplexes [68, 69]. The role of the positive charge is confirmed by studies with an uncharged analogue which showed only a small increase in stability relative to the negatively charged phosphodiester backbone [68]. The triplex binding of such GA-containing phosphoramidates was maintained in millimolar concentrations of potassium, conditions that usually inhibit antiparallel triplex formation as a result of competing structures adopted by the third strand oligonucleotide. These modifications introduce a chiral phosphate, and experiments with stereouniform phosphoramidates have shown that, although one stereoisomer binds with higher affinity than the simple phosphodiesters, the other binds with lower affinity [68].

Other studies have included a positive charge into the backbone by attaching an aminoethoxy moiety to the 2'-position, Fig. (**4c**) [70, 71].This modification causes a dramatic increase in affinity and association rate. Extension of the side chain by an additional methylene group, using a 2'-aminopropoxy group, caused a significant decrease in triplex stability, suggesting that the protonated group is ideally positioned to interact with a nearby phosphate group [70]. Indeed an NMR structure of an intramolecular triplex containing this modification in the Hoogsteen strand has shown contacts between the amino protons of the aminoethoxy group and the phosphates of the purine strand of the duplex [71].

We have also shown that propargylamino-dU, Fig. (**4d**) in which a positively charged group is added to the 5'-position of thymine causes a dramatic increase in triplex stability [72]. The degree of stabilization was pH dependent, confirming the importance of the positive charge. In this case the stabilization is due to more than inclusion of the positive charge since propylamino-dU had a much lower effect on stability.

Improved Base Stacking

The observation that the increased stability of $^{5Me}C^+$•GC relative to C^+•GC is caused by the extra spine of methyl groups within the DNA major groove leads to the suggestion that other analogues with improved base stacking might form more stable triplexes. The methyl group at the 5-position of thymine is also known to contribute to the stability of the T•AT triplet and it has been shown that the ranking order for 5-substituted derivatives is $^{Br}U > T > U$, suggesting that a bulky substituent can improve activity [24]. A similar effect is seen with 5-propynyl-U, Fig. (**5a**), which also increases triplet stability [73-75], though 5-propynyl-C produces a less stable triplet since this substitution further reduces the pK of N3 to 3.3. The increased stability of 5-propynyl substituted bases is presumably due to increased base stacking interactions.

Other studies have attempted to improve base stacking by adding a extra aromatic rings to thymine. One of the first such analogues to be tested was a pyrido[2,3-*d*]pyrimidine derivative (F) [76]. This base selectively recognises AT base pairs, forming the F•AT triplet, utilising the tautomer shown in Fig. (**5b**). However, these complexes were no more stable than those containing T•AT triplets. In addition experiments examining the cooperative binding of adjacent oligonucleotides showed that stacking of F on F was less favourable than T on T. Similarly quinazoline-2,4(1*H*,3*H*)-dione [77, 78], Fig (**5c**), benzo[*f*]quinazoline-2,4-(1*H*,3*H*)-dione, Fig. (**5d**) [79] and benzo[*g*]quinazoline-2,4-(1*H*,3*H*)-dione Fig. (**5e**) [79] have been examined as thymine analogues. Although each of these analogues selectively recognized AT base pairs, in both parallel and antiparallel triplexes, the complexes were less stable than those containing standard T•AT triplets. Taken together these results suggest that modification of pyrimidine bases to form extended ring systems with

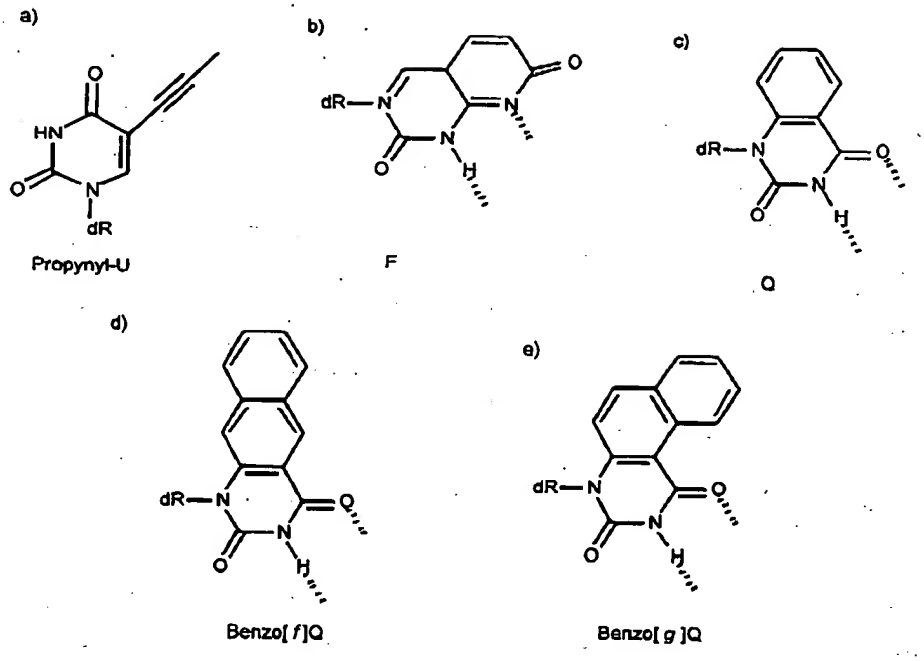

Fig. (5). Base analogues designed to improve third strand base stacking. a) propynyl-dU [73-75], b) pyrido[2,3-*d*]pyrimidine derivative (F) [76], c) quinazoline-2,4(1*H*,3*H*)-dione [77, 78], d) benzo[*f*]quinazoline-2,4-(1*H*,3*H*)-dione, [79], e) benzo[*g*]quinazoline-2,4-(1*H*,3*H*)-dione [79].

the object of increasing the base stacking interactions does not necessarily produce more stable triplex formation. This could either be because the structural parameters of adjacent base triplets do not allow favourable overlap of the additional aromatic rings, or because the increased third strand base stacking disrupts the normal helical parameters, leading to less stable complexes.

Backbone Modifications

RNA

Most triplex studies have examined the formation of triple helices in which all three strands contain DNA, or in which two pyrimidine-containing DNA strands bind to a single stranded RNA (oligo-clamp). There have been a fewer studies on other triplexes containing various combinations of RNA and DNA strands [80-84]. Antiparallel (R•RY) triplexes cannot form if any strand is RNA [80]. In contrast parallel triplexes can be formed with various combinations, though a DNA third strand does not bind against a duplex containing RNA in the purine strand [82, 84]. In general DNA third strands are best for recognizing duplexes with DNA in the purine strand. However there are very great differences in the measured stabilities of various RNA and DNA containing triplexes in different studies. These large discrepancies have been explained by suggesting that the binding affinities of R or D third strands to double helical nucleic acids are length, sequence composition, pH and salt dependent [82]. On the basis of affinity cleavage patterns it has been suggested that Y•RY triplexes fall into two distinct conformational classes [83] dependent on the nature of the backbone i.e. dY•dRdY, rY•dRdY, dY•dRrY and rY•dRrY, rY•rRdY, rY•rRrY.

Inversion of the stereochemistry at the 2'-OH, generating pyrimidine-containing arabinonucleic acid, permits triplex formation only at duplex targets for which the purine strand is DNA (not RNA), in contrast to an RNA containing third strand which formed triplexes at both DNA, RNA and DNA/RNA hybrids under these conditions [85].

2'-OMe

2'-O-methyl-containing oligonucleotides, Fig. (**6 b**) are attractive agents for triplex formation since they generally produce more stable parallel triplexes than their RNA or DNA counterparts [86-89] and addition of the 2'-O-methyl group confers resistance against cellular nucleases. Irrespective of the duplex combination (DNA or RNA) the 2'-O-methyl group forms a more stable parallel triplex than a DNA-containing third strand [89].

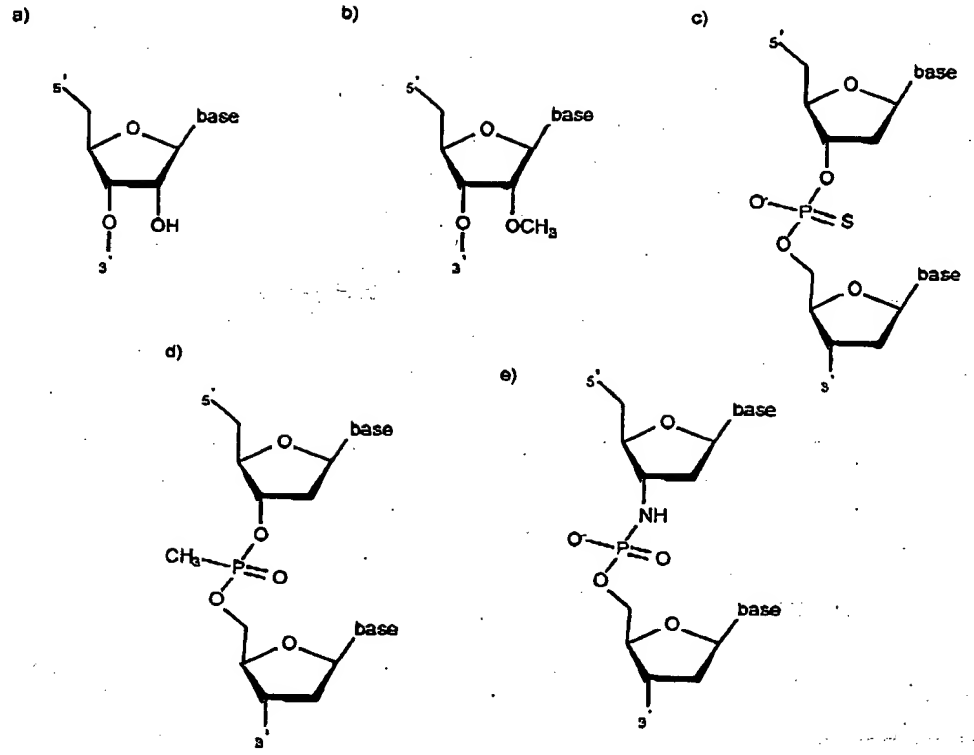

Fig. (6). Modified DNA sugars and backbones for improving triplex stability. a) RNA. b) 2'-OMe-RNA, c) phosphorothioate, d) methylphosphonate, e)N3'→P5' phosphoramidate.

Phosphorothioates

Phosphorothioates, Fig. (**6c**) are promising candidates for oligonucleotide backbone modifications since they possess a similar charge density to normal oligonucleotides, are soluble in aqueous solutions and show reduced degradation by many nucleases. The presence of this group has a dramatic and specific effect on triplex formation. With the phosphorothioate on the purine strand poly(dA-dG).poly(dC-dT) disproportionates into a triplex and single strand, the stability of which increases at lower pHs, while placing the phosphorothioate on the pyrimidine-containing strand inhibits triplex formation [90]. Purine-rich oligonucleotides containing a diastereoisomeric mixture or stereoregular (R_p) phosphorothioate linkages have been shown to form triplexes with similar affinities to phosphodiester linkages oligonucleotides, in contrast to pyrimidine-rich oligonucleotides for which neither diastereomeric or stereoregular (R_p) phosphorothioates formed stable triplexes [91]. GA-containing phosphorothioates form less stable antiparallel triplexes than their phosphodiester counterparts, while a 23-mer GT-containing phosphorothioate did not support triplex formation [92]. For GA-containing third strands it has been shown that inclusion of only two phosphorothioate linkages at each end of a 23-mer oligonucleotide did not reduce the binding affinity, yet was more resistant to nucleases than the phosphodiester oligomer [92]. Antiparallel GA-containing phosphorothioate oligonucleotides have also been shown to inhibit gene transcription *in vivo*. Despite the observation that pyrimidine phosphorothioates do not form parallel triplexes these oligonucleotides have been reported to inhibit T7 polymerase transcription *in vitro* [93].

Methylphosphonates

Nonionic methylphosphonate substitutions, Fig. (**6d**) might be expected to be good candidates for triplex formation since they should overcome the charge repulsion of the third strand in addition to being resistant to nuclease attack. However, there have been conflicting results with oligonucleotides containing this modified backbone. Kibler-Herzog [94] found that no triplexes were produced with dA.dT mixtures when any of the strands consisted of methylphosphonates, though complexes of lower stability could be produced with strands containing alternating phosphodiesters and methylphosphonates [95]. In contrast it has been reported that the formation of a 2:1 complex of $(CT)_8$

with $(AG)_8$ was similar with methylphosphonate and phosphodiester backbones [96]. In addition purine-rich triplex-forming methylphosphonate oligonucleotides have been used to target mRNA [97]. It has also been shown that 2:1 complexes between methylphosphonate dA and phosphodiester dT or methylphosphonate $d(AG)_8$ and phosphodiester $d(CT)_8$ are more stable than those with phosphodiester third strands [98] for which natural $d(AG)_8.d(AG)_8.d(CT)_8$ could not be observed. These complexes also required lower concentrations of cations than normally needed for triplex formation [98]. The affinity of methylphosphonate oligonucleotides may be further increased by inversion of the anomeric configuration from to [99]. In each of these instances the methylphosphonates contained mixtures of R and S-diastereoisomers at the chiral phosphate centre. It remains to be seen whether stereochemically pure methylphosphonates will have better understood triplex forming properties.

N3'→P5' Phosphoramidates

N3'→P5' phosphoramidates, Fig (**6e**) in which O3' is replaced by NH, represent a further modification of the phosphodiester backbone which has been examined for triplex formation. Parallel triplexes containing this modification in the third strand are much more stable than those formed with oligonucleotides with a natural phosphodiester backbone [100,101]. This modification permited stable triplex formation at a target site containing six adjacent guanines at pH 7.0, and was more effective than either ^{5Me}C or tethering an acridine to the 5'-end [100]. By combining the N3'→P5' phosphoramidate backbone with ^{5Me}C the complexes were further stabilized. Phosphoramidate-containing oligonucleotides have been shown to inhibit transcription at pH 7.0 *in vitro*, under conditions in which the natural phosphodiesters have no effect [102]. The avidity of these modified oligonucleotides for forming duplexes and triplexes is such that phosphoramidate dT_{15} partially displaces the dT_{15} stand from a $dT_{15}.dA_{15}$ duplex.[101]. The stability may be further increased by using RNA phosphoramidate oligonucleotides [103]. The reason for the stabilization afforded by N3'→P5' phosphoramidates is not clear, but most likely results form the altered configuration of the backbone, which favours triplex formation [100].

Triplex Binding Ligands

A further means for stabilizing intermolecular DNA triplexes is to use compounds which bind selectively to triplex and not duplex DNA. The first triplex-binding ligands were well known duplex intercalators such as ethidium [104], which preferentially bound to duplex DNA. More recently polycyclic compounds have been developed which selectively bind to triplex and not duplex DNA. The structure of several such agents are shown in Fig. (**7**). The first of these to be described was the benzo[*e*]pyridoindole (BePI) [105], which binds to triplex DNA by intercalation [106]. This agent, together with most other triplex ligands, selectively stabilizes regions of T•AT triplets rather than C^+•GC, presumably because the positive charge on the protonated cytosine prevents binding of the cationic ligand. The selectivity for triplex DNA is thought to arise from the large ring structure, which shows good overlap with the surrounding triplets and which is too large to stack efficiently into duplex DNA. On the basis of molecular modelling studies the side chain of BePI is thought to lie in the major groove between the duplex pyrimidine strand and the third strand (the Watson-Hoogsteen groove) [106]. Several derivatives of BePI have been evaluated for triplex binding activity by Hélène and coworkers who have shown similar good activity with benzo[*g*]pyridoindoles (BgPI) [107]. The side chains of BePI and BgPI have different effects on their activity; for BePI the chain decreases triplex formation whereas it is important for the action of BgPI. It has been suggested that with BgPI these lie in the major groove between the purine strand of the duplex and the third strand (the Crick-Hoogsteen groove) [107]. BgPI possesses a more linear structure than the crescent shaped of BePI, suggesting that the lower base stacking must be compensated by important electrostatic interactions. In contrast benzo[*f*]pyridoindole does not stabilise triplexes. Although most studies have examined the interaction of triplex-binding ligands with parallel triplexes BePI has also been shown to stabilize GT-containing triplexes designed to bind in the antiparallel (but not parallel) orientation [108]. Further studies on related fused aromatic systems have also shown that benzo[*f*]pyridoquinoxaline is also a selective triplex-binding ligand [109]. This series of ligands has been extended to include five membered ring systems such as benzo[*f*]quinoquinoxaline [110] and dibenzophenanthrolines [111]. The latter appears to be the most effective triple-binding ligand in this series described to date. The mono-substituted dibenzophenanthroline derivatives are more active than their disubstituted analogues.

A further compound which has been shown selectively to stabilise triplexes is the berberine alkaloid coralyne [112, 113], Fig. (**7**) which contains four fused aromatic rings. Early studies suggested that this ligand was effective at both C^+•GC and T•AT, though this has since been disputed [114]. Our recent studies have shown that a series of 2,6 disubstituted amidoanthraquinones successfully stabilised triplexes while their 1,4 counterparts only bound to duplex DNA [115]. The different properties of the two series of

compounds are thought to arise from differences in the stacking of the base triplets with the chromophore, combined with DNA groove accessibility. Confirmation of this binding selectivity and a thermodynamic basis for the different triplex/duplex preferences of the 2,6 and 1,4 isomers was provided by calorimetric and spectrophotometric techniques [116]. This study revealed that the 1,4 compounds destabilize DNA triplexes, leading to dissociation of the third strand, whereas their 2,6 isomeric analogues bind to and stabilize triple-stranded structures. A related study has also shown that selective stabilization of triplex DNA can be achieved with a series of disubstituted anthraquinone sulfonamides and suggested that 2,7-functionalised compounds are more potent than their 2,6 counterparts [117]. We have also compared the activity of four di-substituted and two monosubstituted amidoanthraquinone compounds, and have examined how the position of the cationic substituents affects triplex affinity [118]. Triplex affinity for the different substituent patterns decreases in order 2,7 > 1,8 = 1,5 > 2,6, with the equivalent monosubstituted compounds being at least an order of magnitude less efficient.

Naphthylquinoline derivatives, Fig (7) also selectively bind to triplex DNA by intercalation [119-123]. These compounds possess a large aromatic area to stack with the three bases in the triplex, yet, since the aromatic portions are not fused, they possess torsional flexibility and can accommodate the propeller twist of the triplets in which the three bases may not be coplanar. These compounds are also selective for T•AT over C$^+$•GC and are more effective at parallel than antiparallel triplexes [121]. A recent study has also suggested that a naphthylquinoline dimer may bind by bis-intercalation [124].

Triplex-binding ligands have also been used for increasing the strength of binding to sites which contain pyrimidine interruptions. In general these ligands do not affect the stringency of triplex formation, and the relative binding strengths of different oligonucleotide substitutions are not affected [122]. However by increasing the strength of binding, by up to 1000-fold, complexes can be formed at sequences for which there are no clear rules. The naphthylquinoline derivatives have been shown to promote the formation of triplexes at sites containing up to three consecutive base pair inversion using T•CG and G•TA triplets [125]. Studies with BePI have shown that the least destabilizing triplets are the same in both the presence and absence of the ligand and that the third strand base is less important in the presence of the ligand [126]. However addition of the ligand does provide some discrimination between different inverted base pairs. In particular 3-nitropyrrole discriminates CG from GC, TA and AT pairs in the presence, but not the absence of BePI [126]. Duplex regions of $(AT)_n$ have also been targeted with GT-containing oligonucleotides in the presence of the naphthylquinoline triplex-binding ligand, generating complexes containing alternating G•TA and T•AT triplets [127]. Although blocks of alternating T•AT and G•TA triplets alone are not stable, even in the presence a triplex-binding ligand, these complexes can be stabilized by attaching this region to a block of consecutive T•AT triplets, to which the ligand is thought to bind. In this way the duplex $A_{11}(AT)_6.(AT)_6T_{11}$ can be targeted with the third strand $T_{11}(TG)_6$, though this interaction still requires the presence of Mn^{2+} or a triplex binding ligand. These complexes can be extended to longer $(AT)_n$ tracts (up to n = 11) [128] and can be stabilised with shorter T•AT tracts. It should also be noted that these complexes can be further stabilized by including a few C$^+$•GC triplets in the anchoring tail and form in the presence of Mg^{2+}, without addition of a stabilizing ligand [128].

Tethered DNA Binding Agents

One of the first methods to be attempted for increasing triplex stability was to attach a DNA binding ligand to one end of the oligonucleotide. In this way sequence selectivity is achieved by the triplex forming oligonucleotide while the ligand acts as a non-selective anchor increasing the affinity. Most of these studies have used duplex intercalators as DNA binding ligands, though some recent examples have employed triplex binding ligands.

Intercalators

Several groups have shown that covalent attachment of an acridine moiety (usually [2-methoxy,6-chloro,9-amino]acridine) to the end of an oligonucleotide significantly increases triplex stability [129-133]. The acridine is proposed to intercalate at the triplex-duplex junction. Attachment to the 5'-end has a greater effect than at the 3'-end [130, 134], possibly reflecting a structural difference between the 5'-and 3'-triplex-duplex junctions, since the junction at the 5'-end of the third strand is thought to present a strong intercalation site [135, 136]. Most of these studies have used parallel, pyrimidine-containing oligonucleotides, though they have also been used to stabilise antiparallel GA and GT-containing triplexes [92, 137-140] as well as alternate strand triplexes containing both parallel and antiparallel motifs [141]. Attachment generally increases triplex stability by at least 100-fold, though the interaction with related non-cognate sequences is also increased [133]. However attachment of the acridine does not seem substantially to compromise the sequence specificity of binding [139]. At secondary sites where more than one binding

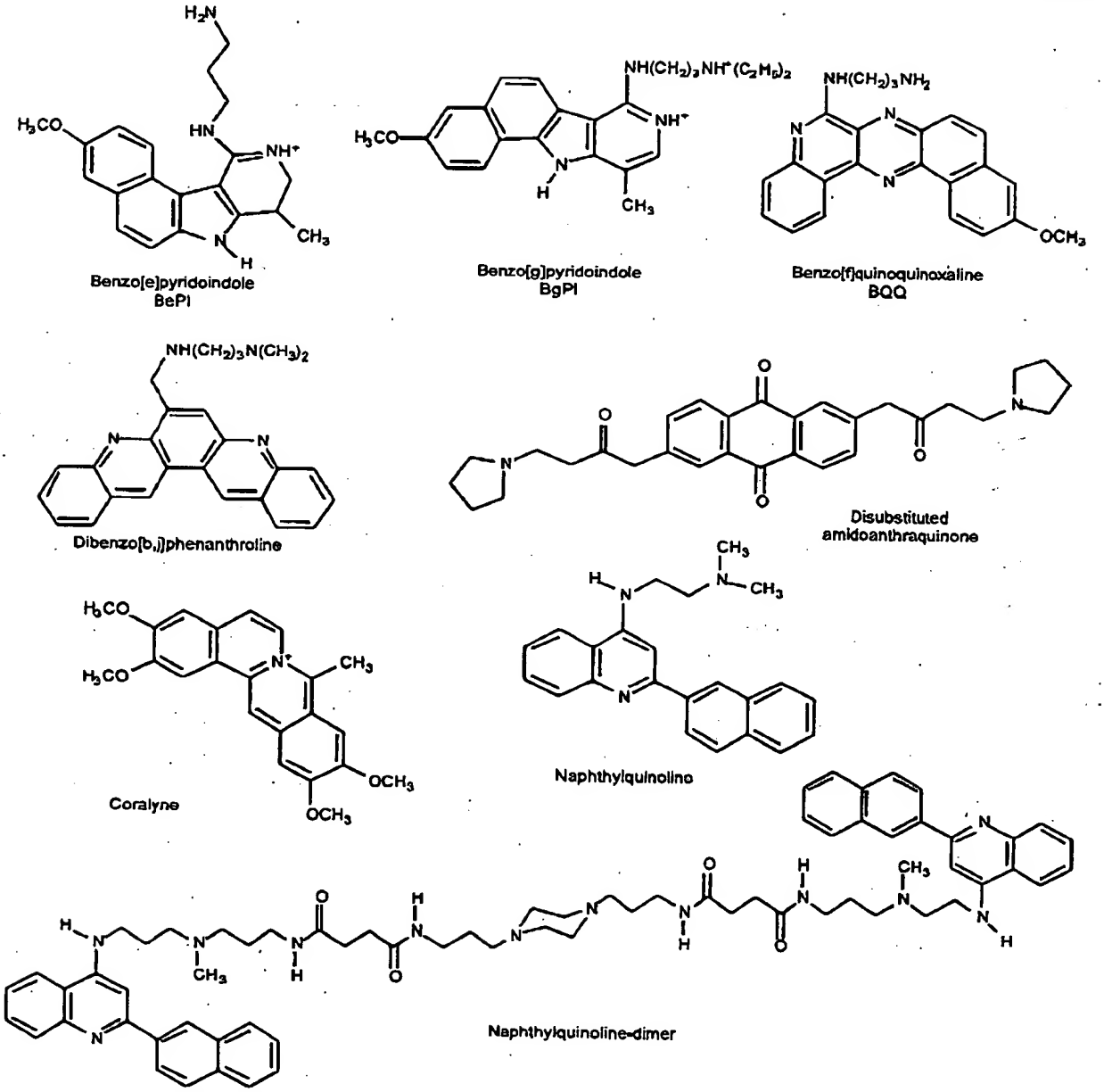

Fig. (7). DNA triplex-binding ligands.

mode is possible the oligonucleotide appears to bind so as to position the mismatches furthest from the intercalating moiety [133]. For GA-containing oligonucleotides covalent attachment of acridine partially overcomes the inhibitory effect of potassium on triplex formation [138], though GA containing oligonucleotides are still superior to those containing GT-residues [139].

Other intercalating agents which have been successfully linked to third strand oligonucleotides include orthophenanthroline [135], anthraquinone derivatives [142] and ellipticine [143].

Cross-linking Agents

The stability of DNA triplexes can also be increased by tethering a chemically reactive group to one or other end of the third strand oligonucleotide. The earliest experiments of this type used azidoproflavin [4] or azidophenacyl [144] to form monofunctional adducts. Bifunctional DNA cross-links can be formed upon long

wavelength irradiation of psoralen, conjugated to an oligonucleotide via either its 4' or 5 position, and can be used as a means of covalently attaching a triplex-forming oligonucleotide to its target site. This not only achieves selective gene inactivation, but provides a means for delivering a tethered mutagen to a target site, for the introduction of site-specific DNA damage. The most reactive sequence for photocrosslinking by psoralen is TpA, linking the thymines across the two strands [145]. Psoralen-linked oligonucleotides are therefore effective at triplex sites which are bordered by a TpA step. The first experiments of this type attached psoralen to the 5'-end of pyrimidine-containing third strand oligonucleotides [146, 147]. In this way successful complex formation was demonstrated at a 16 base oligopurine tract in the HIV proviral sequence at pH 6.0, even though this target sequence contained a run of six contiguous GC base pairs. However at elevated oligonucleotide concentrations secondary binding was observed at a site containing only eight matching base pairs, suggesting that the covalent reaction might induce the formation of some non-productive complexes. Binding was further increased by replacing the third strand cytosines with guanines forming parallel G•GC triplets. Similarly a 20 base pair sequence within the aromatase gene has been targeted with a psoralen-linked oligonucleotide, even though this polypurine tract is interrupted by three CG base pairs, for which T•CG triplets were employed [148, 149].

Psoralen-linked GA-containing oligonucleotides have also been used to introduce a mutation into a selected triplex target sequence. Most of the mutations detected are TA → AT transversions and are targeted at the psoralen intercalation site [151-152]. Mutations are generated by transcription-coupled repair [153] and involve the error-prone repair pathway, either alone or in combination with nucleotide excision repair [154]. This mechanism may depend on the sequence context since other studies have shown that a 19-mer GT-containing oligonucleotide with a 5'-psoralen is not repaired [155]. Double psoralen adducts, located at both ends of a triplex-forming oligonucleotide cannot be repaired efficiently in human cells [156]. The efficiency of triplex formation and gene inactivation can be further increased by tethering an acridine to the other end of the oligonucleotide [157].

Other DNA-binding agents which have been successfully tethered to triplex-forming oligonucleotides include the AT-selective minor groove binding ligand Hoechst 33258 [158, 159], the topoisomerase I inhibitor camptoethecin [160], duocarmycin [161], the DNA cleavage agent bleomycin [162] and a platinum-containing DNA cross-linking agent [163].

Tethered Triplex-binding Ligands

Benzopyridoindole and benzopyridoquinoline triplex-binding ligands have been attached to either the 5'-end or to an internucleotide position of triplex-forming oligonucleotides [164, 165]. For internal attachment the ligands were incorporated as an additional nucleotide, rather than replacing an existing one. All the derivatives stabilised triple helical structures more than either attachment of an acridine moiety or addition of the free ligand at an equivalent concentration. Comparison of their effect on two different parallel triplexes showed that the attached ligand had a greater stabilising effect on the sequence containing a longer run of contiguous thymines. Benzo[*h*]quinoquinoxaline was the most effective compound when attached to the 5'-end of the third strand, while BePI was the best ligand for the internal site. In addition these modifications did not alter the stringency of triplex formation, and introduction of a single mismatch caused a large decrease in binding affinity. We have also prepared oligonucleotides with a naphthylquinoline-derivative tethered to the 5'-end and have shown that these successfully stabilise triplexes to a greater extent than an equivalent concentration of free ligand [166]. It should also be noted, however, that with the tethered complex there is only one ligand stabilising each triplex, whereas with the free ligand several molecules may be able to bind simultaneously at different positions along the triplex.

Inclusion of a duplex binding agent within the third strand oligonucleotide has also be used to increase binding to sites containing pyrimidine interruptions, by placing an internal acridine group adjacent to the base facing the inverted pyrimidine.purine base pair [167, 168]. In this way, the loss of triplex stability at the inversion is partly overcome by the additional binding free energy of the intercalator, in a similar fashion to that achieved by attaching an intercalator to the 5'-end of the third strand. In the absence of a base inversion inclusion of an internal acridine has little or no effect on triplex stability, possibly because it is a duplex- rather than a triplex-specific intercalator. For targets containing CG or TA inversions the acridine moiety increases the stability of triplexes with either natural or synthetic bases opposing the pyrimidine base. Recognition of the TA base pair is strongest using either acridine or propanediol with an acridine on its 3'-side. Recognition of CG is greatest with either cytosine with acridine on its 3'-side or guanine with acridine on its 5'-side [167]. Similarly acridine-conjugated oligonucleotides have been used to stabilize the formation of triplexes at oligopurine tracts containing pyrimidine interruptions [140]. In these studies various natural or synthetic base analogues were placed opposite the pyrimidine residue, while the acridine was tethered to the 5'-end. These base 'mismatches' had a

greater destabilizing effect when place close to the intercalator end of the oligonucleotide.

Antiparallel Triplexes

Since pyrimidine-containing oligonucleotides, forming parallel DNA triplexes, require conditions of low pH, necessary for protonation of the third strand cytosine, it might be expected that purine-containing oligonucleotides designed to for antiparallel triplexes would be superior. Nonetheless these have not been so widely used for several reasons. Firstly the stability of antiparallel triplexes seems to vary widely from one study to another. Secondly these structures are dominated by G.GC, which usually constitutes over 60% of the triplets present, and G-rich oligonucleotides are known to adopt unusual structures which compete for triplex formation. Thirdly there are fewer studies on the recognition of pyrimidine interruptions and there is no specific means of recognising TA (see below).

Stability

In some studies antiparallel triplexes are shown to have very high affinities; short oligonucleotides exhibit nanomolar binding constants [169, 170], while in other studies longer oligonucleotides, under similar conditions, fail to show binding at micromolar concentrations [137, 170-173]. The simplest explanation for this differences is that the formation of antiparallel triplexes is very sequence dependent. This may be due to the different positions of the third strand backbone in G•GC, A•AT and T•AT triplets; unlike parallel T•AT and C^+•GC triplets which are isohelical. The most stable complexes are likely to be those with the lowest number of ApG and GpA steps in the homopurine target site. This sequence dependence is further compounded by the observation that the affinity of antiparallel triplexes is dominated by the G•GC triplet, which imparts a much greater stability that either A•AT or T•AT [137]. However, G-rich oligonucleotides form strong inter- and intramolecular structures, possibly involving G-quartet formation, which reduce the effective concentration of the oligonucleotide by competing with triplex formation. The best antiparallel triplexes may therefore be those in which the third strand has a high G-content, but in which the guanines are arranged in such a way as to prevent the formation of unusual structures.

There have been few studies directly comparing the stability of triplexes formed with CT- GT- and GA-containing oligonucleotides. It is generally agreed that GT triplexes are less stable than their GA counterparts [171, 174-176], possibly because the antiparallel G•GC triplet is more closely isomorphous to A•AT than reverse Hoogsteen T•AT [11]. The kinetics of triplex formation are also faster for antiparallel than parallel complexes [174-177].

Length

Although triplex stability generally increases with the length of the third strand, this is not necessarily the case for antiparallel triplexes. Cheng & Van Dyke [178] showed that, for the GT-motif short third strands (12-mers) could produce more stable complexes than longer oligonucleotides and that, in general, extensions and mutations at the 3'-end had greater effect than those at the 5'-end. Based on this observation they suggested that antiparallel triplex formation proceeded in the 3'→5' direction. Similarly in a REPSA selection assay for oligonucleotides which generate stable triplexes at a 19-mer target site, perfect matches were observed for the 13 bases at the 3'-end, while mismatches were selected at the 5'-end, and shorter oligonucleotides generated complexes with much reduced stability [179]. The greater effect of the 3'-end of the third strand has also been observed in footprinting experiments with oligonucleotides containing $(GGA)_n$ repeats, for which slipped structures were observed [171]. More recently this effect has been confirmed by Arimondo *et al.* [180] who compared the strength of triplex formation by $(AGG)_4$ and $(GGA)_4$. Although both complexes contained 4 x A•AT and 8 x G•GC triplets the oligonucleotide with a 3'-G [*i.e.* $(AGG)_4$] bound about 6-times better than the one with a 3'-A [*i.e.* $(GGA)_4$]. The addition of A•AT and G•GC triplets to the 5'-end of $(AGG)_4$ caused an increase in triplex stability as expected. In contrast, although the addition of a single G to the 3'-end of $(GGA)_4$ increased stability by about 10-fold, the addition of further As caused a decrease in stability. Furthermore overhanging mismatched bases had a much greater effect on stability when added to the 3'- than the 5'-end. Taken together these results suggest that antiparallel triplex formation proceeds from the 3'-end of the third strand and that nucleation is more efficient when the first base is a guanine.

Self Association

G-rich oligonucleotides are known to be able to form stable G-quartet structures, which compete for triplex formation in the presence of physiological concentration of monovalent cations, particularly K^+ [181, 182]. In addition GA- (but not GT) containing oligonucleotides can form homoduplexes, stabilised by internal GA repeats [183, 184]. The situation is further complicated by the observation that different inter- or intra-molecular structures can be formed with different sequences of (G,A)-containing oligonucleotides [185].

Self association of these oligonucleotides can effectively compete with triple helix formation and prevent complete triplex formation since, at high concentrations, more of the oligonucleotide is trapped in self-associated structures. The self-structures adopted by GA-containing oligonucleotides explain the opposite temperature dependence of triplexes formed with GA- and GT-containing oligonucleotides. The binding of GT-oligonucleotides decreases on raising the temperature from 4 to 37°C, in contrast to GA oligonucleotides for which the binding was increased, as the self-associated structure become less stable [183, 184].

Development of conditions which decrease the ability of G-containing oligonucleotides to form unusual structures will greatly improve our understanding of the formation of antiparallel triplexes. Phosphorothioate GA-containing oligonucleotides form less stable self-associated structures [92], but these form less stable triplexes than their phosphdiester counterparts. Divalent metal ions also alter the aggregation of G-rich sequences. Divalent metal ions, usually magnesium, are essential for forming antiparallel triplexes, though their effect is inhibited by physiological concentrations of K^+. However, the competition between triplex and self-associated structures is altered with other transition metal ions, and Co^{2+}, Mn^{2+}, and Ni^{2+} can promote triplex formation [186]. Other studies have attempted to overcome this problem by using base analogues which retain the ability to form Reverse-Hoogsteen hydrogen bonds, but which can no longer self associate. Replacement of guanine N7 with carbon, forming 7-deazaguanine, eliminates the formation of G-quartets, but decreases the ability to form triplexes [187]. Similarly several studies have replaced the guanine O6 with sulphur using 6-thioguanine [182, 188, 189]. This also reduces the binding affinity of the third strand, though it permits triplex formation in the presence of 200 mM K^+. An alterative strategy using 7-deazaxanthine (c^7X) in place of A or T in antiparallel oligonucleotides also achieved high affinity triplex formation under physiological potassium concentrations [187, 190].

Another approach uses the formation of a short duplex at either the 3'- or 5'-end of the third strand to prevent self association of GA oligonucleotides [191]. In these studies a 20-mer third strand was annealed with 10-17-mer oligonucleotides which were complementary to one or other end. These 'zipper' oligonucleotides still form a triplex over the entire length of the target site, suggesting that the short complementary strand has become 'unzipped'. This duplex region can include a large portion of the third strand and the minimal exposed single-stranded region can be as short as three nucleotides at the 3'-end or six nucleotide at the 5'-end. Examples of such 'zipper' oligonucleotides are shown in Fig. (8). The shorter length required at the 3'-end is consistent with the suggestion that triplex nucleation occurs in the 3'→5' direction.

Future Prospects

The work described above indicates that an enormous number of DNA analogues have been synthesized as potential triplex agents in the last 10 years. In addition there is an even greater literature on other analogues which have been used for developed as antisense agents. Is it possible to use these studies to define some of the features which are necessary for triplex formation which might guide the design of further derivatives with improved activity? On the basis of the results described above I will suggest possible developments for generating stable parallel, and antiparallel triplexes as well as some more general features which may improve triplex stability. These various modifications are summarised in Fig. (9).

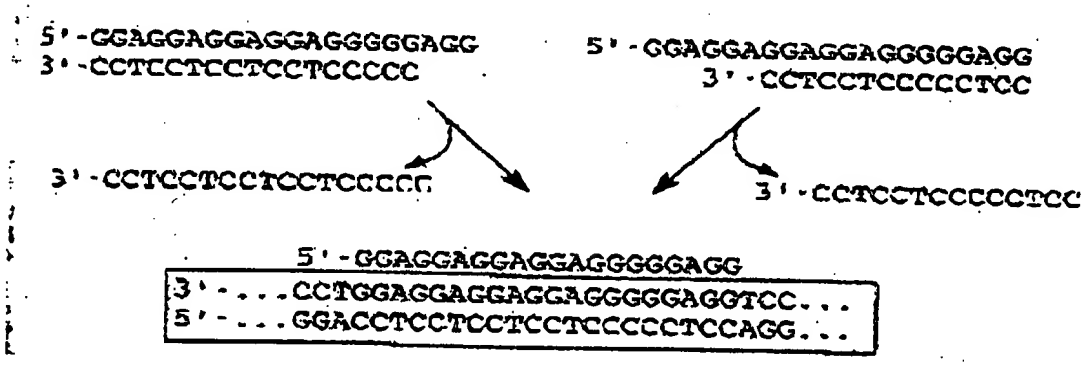

Fig. (8). Examples of 'zipper' oligonucleotides used to prevent self-association of guanine-containing third strands. The sequences were taken from [191].

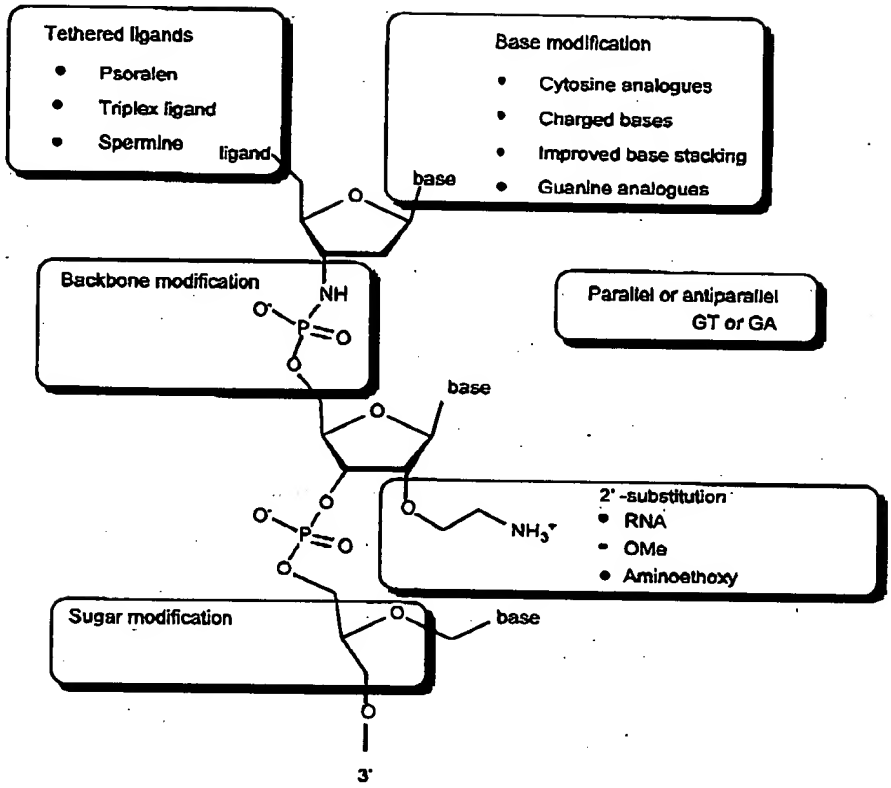

Fig. (9). Summary of potential sites of oligonucleotide modification for generating triplexes with enhanced stability.

Parallel Triplexes

One major problem for generating stable parallel triplexes under physiological conditions is the pH dependency of the C^+•GC triplet. Although several analogues have been shown to alleviate this problem none of these has found widespread use, possibly because of their limited availability, but also because of the large number of choices. In addition although many analogues remove the pH dependency they generate less stable triplexes than those formed with C^+•GC at low pH. Several features seem important in this regard. Firstly, since it is known that C^+•GC is more stable than T•AT [18-21], novel cytosine analogues with a hydrogen bond donor at a position equivalent to N3 should retain the positive charge either on the ring system, as in 2-aminopyridine [38-41], or added to a side group which may be attached to either N4 or C5 [59-62]. The importance of the positive charge suggests that the stability of T•AT might be improved by generating a charged analogue of T. However, since adjacent C^+•GC triplets are known to be destabilising, due to repulsion between the charged groups, it may be necessary to prepare both charged and uncharged analogues of T and C, and determine the optimum arrangement of the charged derivatives. Secondly it is necessary that the X•GC triplet should be isostructural with T•AT so as to minimise any distortions in the third strand backbone between adjacent base analogues. This may not be possible with purine analogues for recognition of GC when used alongside T•AT triplets, and similar derivatives, such as 2-aminopurine [45] or 8-oxoguanine may need to be developed for recognition of AT in these contexts. Thirdly triplex stability may be enhanced by increasing the base stacking in the third strand. However, simple addition of aromatic rings to the pyrimidine nucleus does not seem to be of benefit [76-79], possibly because the improved stacking of the single stranded oligonucleotide hinders triplex formation. Other stacking interactions, such as those involving appended propynyl or propargylamino groups [72-75], may be a better option. Lastly it is possible that different sugars should be used for third strand C or T, since it appears that rigid linkers improve the stability of T•AT, while flexible linkers improve the stability of C^+•GC [35, 36].

Antiparallel Triplexes

There have been fewer studies developing base analogues for use in antiparallel triplets, though there

are several outstanding problems which need to be addressed. Firstly there is the question of whether to use GA- or GT- containing oligonucleotide. GA sequences are more prone to self association [184, 185], yet the A•AT triplet is more closely isostructural to G•GC than T•AT [11]. Since these structures are dominated by the stability of the G•GC triplet, further analogues for isostructural recognition of AT (xanthine) may be useful. Secondly novel analogues (such as 6-thioguanine and 7-deazaguanine) will be needed to overcome the tendency of G-rich oligonucleotides to aggregate [188-190]. These limitations require further work optimizing the choice of third strand oligonucleotide. Thirdly, by analogy with C^+•GC, it may be beneficial to introduce positively charged groups onto the purine ring.

Stability

It is generally agreed that the lower stability of triplexes, relative to duplexes, can in part be attributed to charge repulsion between the three negatively charged phosphodiester backbones. Although several studies have shown that the introduction of positively charged groups into the backbone [59-66], sugar [70, 71] or bases [67-69, 72] can produce stable triplexes, these have usually been used in isolation. It is worth remembering that each triplet carries three negatively charged phosphates; so that yet more stable triplexes may be produced by combining several of these modifications in a single oligonucleotide. A further benefit of these modified oligonucleotides is that they often confer increased biological stability and resistance against nucleases. Triplex binding ligands offer a further method for increasing triplex stability which may also be useful when combined with some of the other approaches, or tethered to the end of the third strand. A further limiting factor for both triplex motifs is that there is still no good method for recognizing pyrimidine interruptions [15, 16]. This is more acute for antiparallel complexes for which there is no method for recognizing a TA base pair. Any novel bases for recognizing TA or CG will also need to be structurally compatible the existing method for targeting GC and TA. Triplex (or duplex) ligands may also be used for stabilizing weaker complexes across targets containing pyrimidine interruptions.

Although there have been many significant advances in the use of triplex forming oligonucleotides there are still many unanswered questions. Resolving these problems will require further close collaborations between chemists and molecular biologists before the potential of this strategy for targeting DNA is fully realised.

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References

[1] Felsenfeld, G.; Rich, A. *Biochim. Biophys. Acta* 1957, 26, 457.

[2] Felsenfeld, G.; Davis, D.R.; Rich, A. *J. Am. Chem. Soc.* 1957, *79*, 2023.

[3] Moser, H.E.; Dervan, P.B. *Science* 1987, *238*, 645.

[4] Le Doan, T.; Perrouault, L.; Praseuth, D.; Habhoub, N.; Decout, J.L.; Thuong, N.T.; Lhomme, J.; Hélène, C. *Nucleic Acids. Res.* 1987, *15*, 7749.

[5] Soyfer, V.N.; Potaman, V.N. *Triple Helical Nucleic Acids* Springer-Verlag: New York, Berlin, 1996.

[6] Thuong, N.T.; Hélène, C. *Angew. Chem. Int. Ed. Engl.* 1993, *32*, 666.

[7] Vasquez, K.M.; Wilson, K.H. *Trends in Biochem.* 1998, *23*, 4.

[8] Chubb, J.M.; Hogan, M.E. *Trends in Biotechnol.* 1992, *10*, 132.

[9] Chan, P.P ; Glazer, P.M. *J. Mol. Med.* 1997, *75*, 267.

[10] Neidle, S. *Anti-Cancer Drug Des.* 1997, *12*, 433.

[11] Radhakrishnan, I.; Patel, D.J. *Structure* 1994, *2*, 17.

[12] Beal, P.A.; Dervan, P.B. *Science* 1991, *251*, 1360.

[13] Chen, F.-M. *Biochemistry* 1991, *30*, 4472.

[14] Radhakrishnan, I.; Patel, D.J. *Structure* 1993, *1*, 135.

[15] Doronina, S.O.; Behr, J.P. *Chem. Soc. Rev.* 1997, *26*, 63.

[16] Gowers, D.M.; Fox, K.R. *Nucleic Acids Res.* 1999, *27*, 1569.

[17] Lee, J.S.; Wordsworth, M.L.; Latimer, L.J.P.; Morgan, A.R. *Nucleic. Acids. Res.* 1984, *12*, 6603.

[18] Roberts, R.W.; Crothers, D.M. *Proc. Natl. Acad. Sci. U.S.A.* 1996, *93*, 4320.

[19] Asensio, J.L.; Lane, A.N.; Dhesi, J.; Bergvist, S.; Brown, T. *J. Mol. Biol.* 1998, *275*, 811.

[20] Keppler, M.D.; Fox, K.R. *Nucleic Acids Res.* 1997, *25*, 4464.

[21] Volker, J.; Klump, H.H. *Biochemistry* 1994, *33*, 13502.

[22] Giovannangeli, C.; Rougée, M.; Garestier, T.; Thuong, N.T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* 1992, *89*, 8631.

[23] Xodo, L.E.; Manzini, G.; Quadrifoglio, F.; van der Marel, G.; van Boom, J. *Nucleic Acids Res.* 1991, *19*, 5625.

[24] Povsic, T.J.; Dervan, P.B. *J. Am. Chem. Soc.* 1989, *111*, 3059.

[25] Koh, J.S.; Dervan, P.B. *J. Am. Chem. Soc.* 1992, *114*, 1470.

[26] Singleton, S.F.; Dervan, P.B. *Biochemistry* 1992, *31*, 10995.

[27] Ono, A.; Ts'o, P.O.P.; Kan, L. *J. Am. Chem. Soc.* 1991, *113*, 4032.

[28] Ono, A.; Ts'o, P.O.P.; Kan, L. *J. Org. Chem.* **1992**, *57*, 3225.

[29] Egholm, M.; Christensen, L.; Dueholm, K.L.; Buchardt, O.; Coull, J.; Nielsen, P.E. *Nucleic Acids Res.* **1995**, *23*, 217.

[30] Kuhn, H.; Demidov, V.V.; Frank-Kamenetskii, M.D.; Nielsen, P.E. *Nucleic Acids Res.* **1998** *26*, 582.

[31] Berressem, R.; Engels, J.W. *Nucleic Acids Res.* **1995**, *23*, 3465.

[32] Xiang, G.; Soussou, W.; McLaughlin, L.W. *J. Am. Chem. Soc.* **1994**, *116*, 11155.

[33] Xiang, G.; Bogacki, R.; McLaughlin, L.W. *Nucleic Acids Res.* **1996**, *24*, 1963.

[34] Xiang, G.; McLaughlin, L.W. *Tetrahedron* **1998**, *54*, 375.

[35] Tarkoy, M.; Leumann, C. *J. Org. Chem.* **1993**, 32, 1432.

[36] Jones, R.L.; Swaminathan, S.; Milligan, J.F.; Waswani, S.; Froehler, B.C.; Matteucci, M.D. *J. Am. Chem. Soc.* **1993**, *115*, 9816.

[37] von Krosigk, U.; Benner, S.A. *J. Am. Chem Soc.* **1995**, *117*, 5361.

[38] Bates, P.J.; Laughton, C.A.; Jenkins, T.C.; Capaldi, D.C.; Roselt, P.D.; Reese, C.B.; Neidle, S. *Nucleic Acids Res.* **1996**, *24*, 4176.

[39] Cassidy, S.A.; Slickers, P.; Trent, J.O.; Capaldi, D.C.; Roselt, P.D.; Reese, C.B.; Neidle, S.; Fox, K.R. *Nucleic Acids Res.* **1997**, *25*, 4891.

[40] Hildbrand, S.; Leumann, C. *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1968.

[41] Hildbrand, S.; Blaser, A.; Parel, S.P.; Leumann, C.J. *J. Am. Chem. Soc.* **1997**, *119*, 5499.

[42] Horne, D.A.; Dervan, P.B. *Nucleic Acids Res.* **1991**, *19*, 4963.

[43] Froehler, B.C.; Ricca, D.J. *J. Am. Chem. Soc.* **1992**, *114*, 8320.

[44] Sun, J.-S.; De Bizemont, T.; Duval-Valentin, G.; Monteny-Garestier, T.; Hélène, C. *C.R.Acad. Sci. Paris Serie III* **1991**, *313*, 585.

[45] Rolg, V.; Kurfurst, R.; Thuong, N.T. *TetrahedronLett.* **1993**, *34*, 1601.

[46] Miller, P.S.; Cushman, C.D. *Biochemistry* **1992**, *31*, 2999.

[47] Jetter, M.C.; Hobbs, F.W. *Biochemistry* **1993**, *32*, 3249.

[48] Krawczyk, S.H.; Milligan, J.F.; Wadwani, S.; Moulds, C.; Froehler, B.C.; Matteucci, M.D. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3761.

[49] Brunar H.; Dervan, P.B. *Nucleic Acids Res.* **1996**, *24*, 1987.

[50] Hunziker, J.; Priestley, E.S.; Brunar, H.; Dervan, P.B. *J. Am.Chem. Soc.* **1995**, *117*, 2661.

[51] Koshlap, K.M.; Schultze, P.; Brunar, H.; Dervan, P.B.; Feigon, J. *Biochemistry* **1997**, *36*, 2659.

[52] Koh, J.S.; Dervan, P.B. *J. Am. Chem. Soc.* **1992**, *114*, 1470.

[53] Radhakrishnan, I.; Patel, D.J.; Priestley, E.S.; Nash, H.M.; Dervan, P.B. *Biochemistry* **1993**, *32*, 11228.

[54] Priestley, E.S.; Dervan, P.B. *J. Am. Chem. Soc.* **1995**, *117*, 4761.

[55] St. Clair, A.; Xiang, G.; McLaughlin, L.W. *Nucleosides Nucleotides* **1998**, *17*, 925.

[56] Marfut, J.; Parel, S.P.; Leumann, C.J. *Nucleic Acids Res.* **1997**, *25*, 1875.

[57] Hampel, K.J.; Crosson, P.; Lee, J.S. *Biochemistry* **1991**, *30*, 4455.

[58] Thomas, T.; Thomas, T.J. *Biochemistry* **1993**, *32*, 14068.

[59] Barawkar, D.A.; Kumar, V.A.; Ganesh, K.N. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 1665.

[60] Barawkar, D.A.; Rajeev, K.G.; Kumar, V.A.; Ganesh, K.N. *Nucleic Acids Res.* **1998**, *24*, 1229.

[61] Rajeev, K.G.; Jadhav, V.R.; Ganesh, K.N. *Nucleic Acids Res.* **1997**, *25*, 4187.

[62] Ganesh, K.N.; Rajeev, K.G.; Pallan, P.S.; Rana, V.S.; Barawkar, D.A.; Kumar, V.A. *Nucleosides Nucleotides* **1997**, *16*, 1271.

[63] Nara, H.; Ono, A.; Matsuda, A. *Bioconjugate Chem.* **1995**, *6*, 54.

[64] Tung, C.H.; Breslauer, K.J.; Stein, S. *Nucleic Acids Res.* **1993**, *21*, 5489.

[65] Sund C.; Puri, N.; Chattopadhyaya. J. *Tetrahedron* **1996**, *52*, 12275.

[66] Sund C.; Puri, N.; Chattopadhyaya, J. *NucleosidesNucleotides* **1997**, *16*, 755.

[67] Chaturvedi, S.; Horn, T.; Letsinger, R.L. *Nucleic Acids Res.* **1996**, 24, 2318.

[68] Dagle, J.M.; Weeks, D.L. *Nucleic Acids Res.* **1996**, *24*, 2143.

[69] Bailey, C.P.; Dagle, J.M.; Weeks, D.L. *Nucleic Acids Res.* **1998**, *26*, 4860.

[70] Cuenoud, B.; Casset, F.; Husken, D.; Natt, F.; Wolf, R.M.; Altmann, K.H.; Martin, P.; Moser, H.E. *Angew. Chem. Int. Ed. Engl.* **1998**, *37*, 1288.

[71] Blommers, T.J.J.; Natt, F.; Jahnke, W.; Cuenoud, B. *Biochemistry* **1998**, *37*, 17714.

[72] Biljapur, J.; Keppler, M.D.; Bergqvist, S.; Brown, T.; Fox, K.R. *Nucleic Acids Res.* **1999**, *27*, 1802.

[73] Colocci, N.; Dervan P.B. *J. Am. Chem. Soc.* **1994**, *116*, 785.

[74] Froehler, B.C.; Wadwani, S.; Terhorst, T.J.; Gerrard, S.R. *Tetrahedron Lett.* **1992**, *33*, 5307.

[75] Phipps, A.K.; Tarkoy, M.; Shultze, P.; Feigon, J. *Biochemistry* **1998**, *37*, 5820.

[76] Staubli, A.B.; Dervan, P.B. *Nucleic Acids Res.* **1994**, 22, 2637.

[77] Michel, J.; Toulme, J.-J.; Vercauteren, J.; Moreau, S. *Nucleic Acids Res.* **1996**, *24*, 1127.

[78] Michel, J.; Gueguen, G.; Vercauteren, J.; Moreau, S. *Tetrahedron* **1997**, *53*, 8457.

[79] Godde, F.; Toulme, J.-J.; Moreau, S. *Biochemistry* **1998**, *37*, 13765.

[80] Semerad, C.L.; Maher L.J. *Nucleic Acids Res.* **1994**, *22*, 5231.

[81] Escudé C.; Francois, J.-C.; Sun, J.-S.; Ott, G.; Sprinz, M.; Garestier, T.; Hélène, C. *Nucleic Acids Res.* **1993**, *21*, 5547.

[82] Han, H.; Dervan, P.B. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3806.

[83] Han, H.; Dervan, P.B. *Nucleic Acids Res.* **1994**, *22*, 2837.

[84] Roberts, R.W.;Crothers, D.M. *Science* **1992**, *258*, 1473.

[85] Noronha, A.; Damha, M.J. *Nucleic Acids Res.* **1998**, *26*, 2665.

[86] Wang, S.; Kool, E.T. *Nucleic Acids Res.* **1995**, *23*, 1157.

[87] Shimizu, M.; Konishi, A.; Shimada, Y.; Inoue, H.; Ohtsuka, E. *FEBS Lett.* **1992**, *302*, 155.

[88] Escudé, C.; Sun, J.-S.; Rougée, M.;Garestier, T.; Hélène, C. *C.R. Acad. Sci. Paris Série III* **1992**, *315*, 521.

[89] Kandimalla, E.R.; Venkataraman, G.; Sasisekharan, V.; Agrawal, S. *J. Biomol. Struct. & Dynam.* **1997**, *14*, 715.

[90] Latimer, L.J.P.; Hampel, K.; Lee, J.S. *Nucleic Acids Res.* **1989**, *17*, 1549.

[91] Hacia, J.G.; Wold, B.J.; Dervan, P.B. *Biochemistry* **1994**, *33*, 5367.

[92] Lacoste, J.; Francois, J.-C.; Hélène, C. *Nucleic Acids Res.* **1997**, *25*, 1991.

[93] Alunni-Fabbroni, M.G.; Manfioletti, G.; Manzini, G.; Xodo, L.E. *Eur. J. Biochem.* **1996**, *226*, 831.

[94] Kibler-Herzog, L.; Kell, B.; Zon, G.; Shinozuka, K.; Mizan, S.; Wilson, W.D. *Nucleic Acids Res.* **1990**, *18*, 3534.

[95] Kibler-Herzog, L.; Zon, G.; Whittier, G.; Mizan, S.; Wilson, W.D. *Anti-Cancer Drug Des.* **1993**, *8*, 65.

[96] Callahan, D.E.; Trapane, T.L.; Miller, P.S.; Ts'o, P.O.P.; Kan, L.-S. *Biochemistry* **1991**, *30*, 1650.

[97] Reynolds, M.A.; Arnold, L.J.; Almazan, M.Y.; Beck, T.A.; Hogrefe, R.I.; Metzler, M.D.; Stoughton, S.R.; Tseng, B.Y.; Trapane, T.L.; Ts'o, P.O.P.; Woolf, T.M. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 12433.

[98] Trapane, T.L.; Hogrefe, R.I.; Reynold, M.A.; Kan, L.S.; Ts'o, P.O.P. *Biochemistry* **1996**, *35*, 5485.

[99] Debart, F.; Meyer, A.; Vasseur, J.J.; Rayner, B. *Nucleic Acids Res.* **1998**, *26*, 4551.

[100] Escudé, C.; Giovannangeli, C.; Sun, J.-S.; Lloyd, D.H.; Chen, J.-K.; Gryaznov, S.M.; Garestier, T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4365.

[101] Zhou-Sun, B.W.; Sun, J.S.; Gryaznov, S.N.; Liquier, J.; Garestier, T.; Hélène, C.; Taillandier, E. *Nucleic Acids Res.* **1997**, *25*, 1782.

[102] Giovannangeli, C.; Perrouault, L.; Escudé, C.; Gryaznov, S.; Hélène, C. *J. Mol. Biol.* **1996**, *261*, 386.

[103] Gryaznov, S.M.; Winter, H. *Nucleic Acids Res.* **1998**, 26, 4160.

[104] Scaria, P.V.; Shafer, R.H. *J. Biol. Chem.* **1991**, *266*, 5417.

[105] Mergny, J.L.; Duval-Valentin, G.; Nguyen, C.H.; Perrouault, L.; Faucon, B.; Rougée, M.; Montenay-Garestier, T.; Nisagni, E.; Hélène, C. *Science* **1992**, *256*, 1681.

[106] Pilch, D.S.; Waring, M.J.; Sun, J.-S.; Rougee, M.; Nguyen, C.-H.; Bisagni, E.; Garestier, T.; Hélène, C. *J. Mol. Biol.* **1993**, *232*, 926.

[107] Escudé, C.; Nguyen, C.H.; Mergny, J.-L.; Sun, J.-S.; Bisagni, E.; Garestier, T.; Hélène, C. *J. Am. Chem. Soc.* **1995**, *117*, 10212.

[108] Escudé, C.; Sun, J.-S.; Nguyen, C.H.; Bisagni, E.; Garestier, T.; Hélène, C. *Biochemistry* **1996**, *35*, 5735.

[109] Escudé, C.; Nguyen, C.H.; Kukreti, S.; Janin, Y.; Sun, J.-S.; Bisagni, E.; Garestier, T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3591.

[110] Marchand, C.; Bailly, C.; Nguyen, C.H.; Bisagni, E.; Garestier, T.; Hélène, C.; Waring, M.J. *Biochemistry* **1996**, *35*, 5022.

[111] Baudoin, O.; Marchand, C.; Teulande, M.-P.; Vigneron, J.-P.; Sun, J.-S.; Garestier, T.; Hélène, C.; Lehn, J.-M. *Chem. Eur. J.* **1998**, *4*, 1504.

[112] Lee, J.S.; Latimer, L.J.P.; Hampel, K.J. *Biochemistry* **1993**, *32*, 5591.

[113] Latimer, L.J.P.; Payton, N.; Forsyth G.; Lee, J.S. *Biochem. Cell Biol.* **1995**, *73*, 11.

[114] Moraru-Allen, A.A.; Cassidy, S.; Alvarez, J.-L.A.; Fox, K.R.; Brown, T.; Lane, A.N. *Nucleic Acids Res.* **1997**, *25*, 1890.

[115] Fox, K.R.; Polucci, P.; Jenkins, T.C.; Neidle, S. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7887.

[116] Haq, I.; Ladbury, J.E.; Chowdhry, B.Z.; Jenkins, T.C. *J. Am. Chem. Soc.* **1996**, *118*, 10693.

[117] Kan, Y.Z.; Armitage, B.; Schuster, G.B. *Biochemistry* **1997**, *36*, 1461.

[118] Keppler, M.D.; Read, M.A.; Perry, P.J.; Jenkins, T.C.; Trent, J.O.; Reszka, A.P.; Neidle, S.; Fox, K.R. *Eur. J. Biochem.* **1999**, *263*, 817.

[119] Wilson, W.D.; Tanious, F.A.; Mizan, S.; Yao, S.; Kiselyov, A.S.; Zon, G.; Strekowski, L. *Biochemistry* **1993**, *32*, 10614.

[120] Cassidy, S.A.; Strekowski, L.; Wilson, W.D.; Fox, K.R. *Biochemistry* **1994**, *33*, 15338.

[121] Cassidy, S.A.; Strekowski, L.; Fox, K.R. *Nucleic. Acids Res.* **1996**, *24*, 4133.

[122] Chandler, S.P.; Strekowski, L.; Wilson, W.D.; Fox, K.R. *Biochemistry* **1995**, *34*, 7234.

[123] Strekowski, L.; Gulevich, Y.; Baranowski, T.C.; Parker, A.N.; Kiselyov, A.S.; Lin, S.-Y.; Tanious, F.; Wilson, W.D. *J. Med. Chem.* **1996**, *39*, 3980.

[124] Keppler, M.D.; Zegrocka, O.; Strekowski, L.; Fox, K.R. *FEBS Lett.* **1999**, *447*, 223.

[125] Gowers, D.M.; Fox, K.R. *Nucleic Acids Res.* **1997**, *25*, 3787.

[126] Kukreti, S.; Sun, J.S.; Loakes, D.; Brown, D.M.; Nguyen, C.H.; Bisagni, E.; Garestier, T.; Hélène, C. *Nucleic Acids Res.* **1998**, *26*, 2179.

[127] Chandler, S.P.; Fox, K.R. *FEBS Letts.* **1995**, *360*, 21.

[128] Gowers, D.M.; Fox, K.R. *Nucleic Acids Res.* **1998**, *26*, 3626.

[129] Sun, J.C.; Giovannangeli, C.; Francois, J.C.; Kurfurst, K.; Monteny-Garestier, T.; Asseline, U.; Saison-Behmoaras, T.; Thuong, N.T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6023.

[130] Sun, J.-S.; Francois, J.-C.; Montenay-Garestier, T.; Saison-Behmoaras, T.; Roig, V.; Thuong, N.T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9198.

[131] Birg, F.; Praseuth, D.; Zerial, A.; Thuong, N.T.; Asseline, U.; Le Doan, T.; Hélène, C. *Nucleic Acids Res.* **1990**, *18*, 2901.

[132] Grigoriev, M.; Praseuth, D.; Robin, P.; Hemar, A.; Saison-Behmoaras, T.; Dautry-Varsat, A.; Thuong, N.T.; Hélène, C.; Harel-Bellan, A. *J. Biol. Chem.* **1992**, *267*, 3389.

[133] Stonehouse T.J.; Fox, K.R. *Biochim. Biophys. Acta* **1994**, *1218*, 322.

[134] Montenay-Garestier, T.; Sun, J.S.; Chomilier, J.; Mergny, J.L.; Takasugi, M.; Asseline, U.; Thuoung, N.T.; Rougée, M.; Hélène, C. In: *Molecular Basis of Specificity in Nucleic Acid-Drug Interactions*, B.Pullman & J. Jortner (eds.) Kluwer Academic Publishers. Holland. **1990**, *275*.

[135] Collier, D.A.; Mergny, J.-L.; Thuong, N.T. Hélène, C. *Nucleic Acids Res.* **1991**, *15*, 4219.

[136] Mouscadet, J.-F.; Ketterlé, C.; Goulaouic, H.; Carteau, S.; Subra, F.; Le Bret, M.; Auclair, C. *Biochemistry* **1994**, *33*, 4187.

[137] Fox, K.R. Nucleic Acids Res. **1994**, *22*, 2018.

[138] Gamper, H.B.; Kutyavin, I.V.; Rhinehart, R.L.; Lokhov, S.G.; Reed, M.W.; Meyer, R.B. *Biochemistry* **1997**, *36*, 14816.

[139] Klysik, J.; Kinset, B.M.; Hua, P.; Glass, G.A.; Orson, F.A. *Bioconjugate Chem.* **1997**, *8*, 318.

[140] Orson, F.M.; Klysik, J.; Bergstrom, D.E.; Ward, B.; Glass, G.A.; Hua, P.; Kinesy, B.M. *Nucleic Acids Res.* **1999**, *27*, 610.

[141] Washbrook, E.; Fox, K.R. *Biochem. J.* **1994**, *301*, 569.

[142] Gibson, V.; Anderson, R.J.; Brown, J.R.; Hartley, J.A.; Cassidy, S.A.; Fox, K.R.; Cairns, D. *Pharm. Sci.* **1996**, *2*, 49.

[143] Perrouault, L.; Asseline, U.; Rivalle, C.; Thuong, N.T; Bisagni, E.; Giovannangeli, C.; Le Doan, T.; Hélène, C. *Nature* **1990**, *344*, 358.

[144] Praseuth, D.; Perrouult, L.; Le Doan, T.; Chassignol, M.; Thouong, N.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1349.

[145] Sage, E.; Moustacchi, E. *Biochemistry* **1987**, *26*, 3307.

[146] Giovannangeli, C.; Thuong, N.T.; Hélène, C. *Nucleic Acids Res.* **1992**, *20*, 4275.

[147] Takasugi, M.; Guendouz, A.; Chassignol, M.; Decout, J.L.; Lhomme, J.; Thuong, N.T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5602.

[148] Macaulay, V.M.; Bates, P.J.; McLean, M.J.; Rowlands, M.G.; Jenkins, T.C.; Ashworth, A.; Neidle, S. *FEBS Lett.* **1995**, *372*, 222.

[149] Bates, P.J.; Macaulay, V.M.; McLean, M.J.; Jenkins, T.C.; Reska, A.P.; Laughton, C.A.; Neidle, S. *Nucleic Acids Res.* **1995**, *23*, 4283.

[150] Raha, M.; Wang, G.; Seidman, M.M.; Glazer, P.M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2941.

[151] Wang, G.; Levy, D.D.; Seidman, M.M.; Glazer, P.M. *Mol. Cell. Biol.* **1995**, *15*, 1759.

[152] Gasparro, F.P.; Havre, P.A.; Olack, G.A.; Gunther, E.J.; Glazer, P.M. Nucleic Acids Res. **1994**, *22*, 2845.

[153] Wang, G.; Seidman, M.M.; Glazer, P.M. *Science* **1996**, *271*, 802.

[154] Barre, F.-X.; Giovannangeli, C.; Hélène, C.; Harel-Bellan, A. *Nucleic Acids Res.* **1999**, *27*, 743.

[155] Musso, M.; Wang, J.C.; Van Dyke, M.W. *Nucleic Acids Res.* **1996**, *24*, 4924.

[156] Sandor, Z.; Bredberg, A. *FEBS Lett.* **1995**, *374*, 287.

[157] Duval-Valentin, G.M.; Takasugi, C.; Hélène, C.; Sage, E. *J. Mol. Biol.* **1998**, *278*, 815.

[158] Robles, J.; Rajur, S.;McLaughlin, L.W. *J. Am. Chem. Soc.* **1996**, *118*, 5820.

[159] Rajur, S.B.; Robles, J.; Wiederholt, K.; Kuimelis, R.G.; McLaughlin, L.W. *J. Org. Chem.* **1997**, *62*, 523.

[160] Matteucci, M.; Lin, K.Y.; Huang, T.; Wagner, R.; Sternbach D.D; Mehrotra, M.; Besterman, J.M. *J. Am. Chem. Soc.* **1997**, *119*, 6939.

[161] Lin, C.H.; Patel, D.J. *J. Amer. Chem. Soc.* **1992**, *114*, 10658.

[162] Kane, S.A.; Hecht, S.M.; Sun, J.-S.; Garestier, T.;Hélène, C. *Biochemistry* **1995**, *34*, 16715.

[163] Colombier, C.; Lippert, B.; Leng, M. *Nucleic Acids Res.* **1996**, *24*, 4519.

[164] Silver, G.C.; Nguyen, C.H.; Boutorine, A.S.; Bisagni, E.; Garestier, T.; Hélène, C. *Bioconjugate Chem.* **1997**, *8*, 15.

[165] Silver, G.C.; Sun, J.-S.; Nguyen, C.H.; Boutorine, A.S.; Bisagni, E.; Hélène, C. *J. Am. Chem Soc.* **1998**, *119*, 263.

[166] Keppler, M.D.; McKeen, C.M.; Zegrocka, O.; Strekowski, L.; Brown, T.; Fox, K.R. *Biochem. Biophys. Acta* **1999**, in press.

[167] Zhou, B.; Puga; E.; Sun, J.; Garestier, T.; Hélène, C. *J. Am. Chem Soc.* **1995**, *117*, 10426.

[168] Kukreti, S.; Sun, J.S.; Garestier,T.; Hélène,C. *Nucleic Acids Res.* **1997**, *25*, 4264.

[169] Svinarchuk, F.; Bertrand, J.-R.; Malvy, C. *Nucleic Acids Res.* **1994**, *22*, 3742.

[170] Svinarchuk, F.; Paoletti, J.; Malvy, C. *J. Biol. Chem.* **1995**, *270*, 14068.

[171] Chandler, S.P.; Fox, K.R. *Biochemistry* **1996**, *35*, 15038.

[172] Brown, P.M.; Fox, K.R. *Biochem. J.* **1996**, *319*, 607.

[173] Brown, P.M.; Madden, C.A.; Fox, K.R. *Biochemistry* **1998**, *37*, 16139.

[174] Faucon, B.; Mergny, J.-L.; Hélène, C. *Nucleic Acids Res.* **1996**, *24*, 3181.

[175] Roy, C. *Nucleic Acids Res.* **1993**, *21*, 2845.

[176] Klysik, J.; Kinset, B.M.; Hua, P.; Glass, G.A.; Orson, F.A. *Bioconjugate Chem.* **1997**, *8*, 318.

[177] Paes, H.M.; Fox, K.R. *Nucleic Acids Res.* **1997**, *25*, 3269.

[178] Cheng, A.-J.; van Dyke, M.W. *Nucleic Acids Res.* **1994**, *22*, 4742.

[179] Hardenbol, P.; Van Dyke M.W. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2811.

[180] Arimondo, P.B.; Barcelo, F.; Sun, J.-S.; Maurizot, J.-C.; Garestier, T.; Hélène, C. *Biochemistry* **1998**, *37*, 16827.

[181] Cheng, A.-J.; Van Dyke, M.W. *Nucleic Acids Res.* **1993**, *21*, 5630.

[182] Olivas, W.M.; Maher, L.J. *Nucleic Acids Res.* **1995**, *23*, 1936.

[183] Noonberg, S.B.; Francoise, J.C.; Garestier, T.; Hélène, C. *Nucleic Acids Res.* **1995**, *23*, 1956.

[184] Noonberg, S.B.; Francois, J.-C.; Praseuth, D.; Guieysse-Peugot, A.-L.; Lacoste, J.; Garestier, T.; Hélène, C. *Nucleic Acids Res.* **1995**, *23*, 4042.

[185] Lee, J.S. *Nucleic Acids Res.* **1990**, *18*, 6057.

[186] Blume, S.W.; Guarcello, V.; Zacharias, W.; Miller, D.M. *Nucleic Acids Res.* **1997**, *25*, 617.

[187] Milligan, J.F.; Krawczyk, S.H.; Wadwani, S.; Matteucci, M.D. *Nucleic Acids Res.* **1993**, *21*, 327.

[188] Gee, J.E.; Revankar, G.R.; Rao, T.S; Hogan, M.E. *Biochemistry* **1995**, *34*, 2042.

[189] Rao, T.S.; Durland, R.H.; Seth, D.M.; Myrick, M.A.; Bodpeudi, V.; Revankar, G.R. *Biochemistry* **1995**, *34*, 765.

[190] Faruqi, A.F.; Krawczyk, S.H.; Metteucci, M.D.; Glazer, P.M. *Nucleic Acids Res.* **1997**, *25*, 633.

[191] Svinarchuk, F.; Chemy, D.; Debin, A.; Delain, E.; Malvy, C. *Nucleic Acids Res.* **1996**, *24*, 385.